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Genome of Horsepox Virus

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Here we present the genomic sequence of horsepox virus (HSPV) isolate MNR-76, an orthopoxvirus (OPV) isolated in 1976 from diseased Mongolian horses. The 212-kbp genome contained 7.5-kbp inverted terminal repeats and lacked extensive terminal tandem repetition. HSPV contained 236 open reading frames (ORFs) with similarity to those in other OPVs, with those in the central 100-kbp region most conserved relative to other OPVs. Phylogenetic analysis of the conserved region indicated that HSPV is closely related to sequenced isolates of vaccinia virus (VACV) and rabbitpox virus, clearly grouping together these VACV-like viruses. Fifty-four HSPV ORFs likely represented fragments of 25 orthologous OPV genes, including in the central region the only known fragmented form of an OPV ribonucleotide reductase large subunit gene. In terminal genomic regions, HSPV lacked full-length homologues of genes variably fragmented in other VACV-like viruses but was unique in fragmentation of the homologue of VACV strain Copenhagen B6R, a gene intact in other known VACV-like viruses. Notably, HSPV contained in terminal genomic regions 17 kbp of OPV-like sequence absent in known VACV-like viruses, including fragments of genes intact in other OPVs and approximately 1.4 kb of sequence present only in cowpox virus (CPXV). HSPV also contained seven full-length genes fragmented or missing in other VACV-like viruses, including intact homologues of the CPXV strain GRI-90 D2L/I4R CrmB and D13L CD30-like tumor necrosis factor receptors, D3L/I3R and C1L ankyrin repeat proteins, B19R kelch-like protein, D7L BTB/POZ domain protein, and B22R variola virus B22R-like protein. These results indicated that HSPV contains unique genomic features likely contributing to a unique virulence/host range phenotype. They also indicated that while closely related to known VACV-like viruses, HSPV contains additional, potentially ancestral sequences absent in other VACV-like viruses.

The genus *Orthopoxvirus* includes members of the family *Poxviridae* historically relevant to human health—variola virus (VARV), the etiologic agent of smallpox, and vaccinia virus (VACV), the vaccine virus used to eradicate smallpox (32). Other orthopoxviruses (OPVs), similar to VACV, are zoonotic and significant for human health, including monkeypox virus (MPXV) and cowpox virus (CPXV) (33). Still others, similar to VARV, remain restricted to specific, albeit nonhuman, hosts, including camelpox virus (CMLV) in camels and ectromelia virus (ECTV) in mice. Recent developments have heightened interest in OPV virulence and host range, including the threats of deliberate VARV reintroduction, virulence associated with preemptive smallpox vaccination and use of VACV-based recombinant vaccines, and the introduction of MPXV into the United States (16, 28, 69, 83). Isolation of OPV from infected animals and humans during limited disease outbreaks or from animals in the wild suggests that additional OPVs circulating in nature could represent an emerging disease threat (24, 25, 27, 32, 46, 49, 50, 90).

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Given their importance, OPVs have been extensively studied as models of poxviral molecular biology, genomics, genetics, and virus-host interaction (19, 33, 59). Research has revealed that OPVs contain approximately 170 to 230 genes, with those in central genomic regions generally involved in poxviral intracytoplasmic replication and those in terminal genomic regions involved or potentially involved in virus-host interactions, including manipulation of host immune or cellular apoptotic responses (4, 19, 59, 60, 82, 87).

Comparative analysis of completely sequenced OPV genomes, including most known OPV species and several strains of VARV, VACV and the closely related rabbitpox virus (RPXV), MPXV, CMLV, and CPXV has begun to reveal the degree of variability within the genus *Orthopoxvirus*, verifying that terminal genomic regions are the most variable and thus likely to contribute to the virulence and host range characteristics of different OPVs (2, 9, 21, 22, 36, 39, 51, 52, 54, 58, 78, 80, 81). The precise roles and contributions of many variable genes and gene complements in OPV virulence and host range, however, remain to be fully characterized. It is likely that complete genomic data from uncharacterized OPV isolates will aid in OPV gene identification and functional characterization, while also providing information regarding the pathogenic potential of the virus.

Horsepox virus (HSPV) is an OPV causing horsepox, classically known as a poxviral disease of horses. Although common before the 20th century, horsepox is rare today to the point of being considered extinct (14, 44). Multiple clinical forms of horsepox have been described, including a benign, localized form involving lesions in the muzzle and buccal cavity known previously as contagious pustular stomatitis and a generalized, highly contagious form known as equine papular stomatitis (44, 94). Horsepox has also been associated with an exudative dermatitis of the pasterns described as "grease" or grease heel, a clinical syndrome also associated with other infectious and environmental agents (14, 33, 94). Horsepox is differentiated clinically from two other poxviral diseases of horses, equine molluscum contagiosum and Uasin Gishu disease. Equine molluscum contagiosum is a mild, self-limiting cutaneous disease similar to the human disease and is associated with a virus similar to molluscum contagiosum virus (88, 94). Uasin Gishu disease has been described in nonindigenous horses of eastern Africa and is associated with a poorly characterized OPV; however, generalized skin lesions are proliferative and papillomatous and the disease may be chronic in nature (33, 88, 94). HSPV is yet to be characterized molecularly, with no DNA sequence information available. Given the interest in understanding the genetic basis of viral host range and virulence and the relationships between OPVs, we have sequenced and analyzed the genome of a pathogenic field isolate of HSPV.

MATERIALS AND METHODS

Viral DNA isolation, cloning, sequencing, and sequence analysis. The HSPV strain MNR-76 was isolated from sick horses in Bayan-somon of Khentei aimak, Mongolia, in 1976. MNR-76 causes severe disease in horses of the Mongolian breed, including pyrexia, pustular stomatitis with occasional lesions on udders and ears, and especially severe disease in foals and mares, in which death was noted (S. M. Mamadaliyev, personal communication). Viruses were passaged twice in sheep kidney cells, from which viral genomic DNA was extracted as previously described (93). Random DNA fragments were obtained by incomplete enzymatic digestion with Tsp509I endonuclease, cloned into the dephosphorylated EcoRI site of pUC19 plasmids, and grown in *Escherichia coli* DH10B cells (Gibco BRL, Gaithersburg, Md.). Double-stranded DNA templates were purified and sequenced from both ends with M13 forward and reverse primers using dideoxy chain terminator sequencing chemistries and the Applied Biosystems PRISM 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA). Chromatogram traces were base called with Phred (30), which also produced a quality file containing a predicted probability of error at each base position. The sequences were assembled with Phrap (29) and CAP3 (43) using quality files and default settings to produce a consensus sequence with some subsequent manual editing using the Consed sequence editor (37). Gap closure was achieved by primer walking of gap-spanning clones and sequencing of PCR products. Final DNA consensus sequences represented on average sevenfold redundancy at each base position, contained no obvious polymorphisms, and demonstrated a Consed estimated error rate of less than 0.01 error per 10 kb.

Sequence analysis was conducted essentially as previously described (1). Briefly, DNA composition, structure, repeats, and restriction enzyme patterns were analyzed and open reading frame (ORF) maps created using EMBOSS (70), GCG v.10 (Accelrys, Inc., San Diego, CA), and MacVector (Accelrys, Inc) software packages. ORFs longer than 30 amino acids with a methionine start codon were evaluated for coding potential using the GLIMMER (71) computer program, and those greater than 60 amino acids were subjected to similarity searches against nonredundant protein databases and redundant viral protein databases using BLAST (8) and against viral nucleotide databases using TFASTA and TFASTX (65, 66). Here, 236 ORFs were annotated and numbered from left to right, with alphabetic subordering given to indicate multiple potential fragments of larger OPV ORFs. Given the predicted nature of all HSPV genes and gene products, ORF names were used throughout the text to indicate both the predicted gene and its putative protein product. Genomic, subgenomic, and protein alignments and comparisons were done using DIALIGN v2.2.1 (57) using anchors as generated by CHAOS (17), Multi-LAGAN (18), CLUSTAL W (89), BLAST, FASTA (64), SEAVIEW (34), and DOTTER (84) programs. Phylogenetic analyses were conducted on whole-genome sequences and genomic subregions, including a central region used previously for OPV phylogenetic analysis (positions 26800 to 170171) (22, 51), using PHYLIP (31); PHYLO_WIN (34), TREE-PUZZLE (73), and PHYML (40) programs, with evolutionary models selected using MrModeltest 2.2 (62) and additional analyses conducted on alignments in which poorly aligned regions were removed with Gblocks (20).

Nucleotide sequence accession number. The HSPV MNR-76 genome sequence has been deposited in GenBank under accession no. DQ792504.

RESULTS AND DISCUSSION

Organization of the HSPV genome. HSPV MNR-76 genome sequences were assembled into a contiguous sequence of 212,633 bp. The leftmost nucleotide was arbitrarily designated base 1. Similar to other OPVs, the HSPV genome contained 69% A+T nucleotide composition and a central coding region bounded by two identical inverted terminal repeat (ITR) regions.

HSPV ITRs were 7,527 bp and contained elements similar to repetitive and nonrepetitive sequences characterized in other OPVs, including a portion of the terminal hairpin loop-like sequence (positions 1 to 15 from each terminus) and nonrepetitive region 1 (NR1) (positions 21 to 101 from each terminus) and concatemer resolution (position 21 to 40 from each terminus) sequences identical to those present in VACV strain Copenhagen (CPN) (11, 36, 55). Notably, HSPV lacked extensive tandem repetition of terminally located sequences, containing only single copies of the 69-bp (positions 102 to 170, 100% identical to CPN) and 54-bp (positions 518 to 571, 96% identical to CPN) motifs repeated 8.5 to 42 times in VACV strains and RPXV (9, 10, 36, 51). Incomplete copies of 69-bp (positions 171 to 188), 54-bp (positions 572 to 601), and VACV 125-bp repeat-like (positions 494 to 517) motifs flanked complete 69-bp and 54-bp motifs, which were also separated from each other by an NR2-like sequence (positions 189 to 493, 92% identity to CPN positions 2867 to 3171). The HSPV ITR contained eight ORFs initiating and terminating in the ITR, with HSPV001/HSPV207 encompassing the 54-bp and 125-bp motif region (Table 1). These data indicate that while similar to VACV in regions of the ITR, HSPV organizationally resembles other OPVs such as VARV, MPXV, and ECTV which contain fewer or single complete tandem repeat units in their termini (21, 53, 81).

HSPV contained 236 ORFs potentially encoding proteins of 53 to 1,920 amino acids and sharing similarity with those in previously described OPV genomes (Tables 1 and 2). Of these 236 annotated ORFs, 54 were significantly smaller or fragmented forms of 25 larger ORFs present in other OPVs, leaving 182 potentially full-length OPV gene homologues. The HSPV central genomic region contained genes colinear and highly conserved among other OPV genomes, with ORFs HSPV041 to HSPV145 sharing an average 98% amino acid identity with VACV CPN ORFs F1L to A24R and with CPXV GRI-90 ORFs G1L to A25R (Table 2 and data not shown). Genes in this conserved region included those involved in basic replicative functions such as viral transcription and transcript modification, DNA replication, and assembly of intracellular mature and extracellular enveloped virions (IMVs and EEVs,

TABLE 1. HSPV ORFs in terminal genomic regions compared to best-matching ORFs annotated in VACVs, RPXV, and CPXVs[a]

HSPV ORF	Position (length[b])	VACV[c]											RPXV[d]		CPXV[e]					Putative function/similarity[h]
		CPN			WR		Tian		MVA		mO				GRI			BRI		
		ORF	Length	% Id[f]	ORF	Length	ORF[g]	Length	ORF	Length	ORF	Length	ORF	Length	ORF	Length	% Id[f]	ORF	Length	
Left-terminal genomic region																				
HSPV001	688–473 (72)																	001	64	
HSPV002	1547–804 (248)	C23L	244	86	001	244	C20L	244	**001L**	**136**	L10L	258	001	258	D1L	255	87	003	246	Chemokine binding protein
HSPV003	2723–1676 (349)	**C22L**	**122**	**91**	**002**	**61**			**002L**	**176**	**L09L**	**34**		**63**	D2L	351	95	005	355	TNFR II-like protein, CrmB
					004	**122**					**L08L**	**122**		**122**						
HSPV004	4570–2810 (587)	**C21L**	**113**	**100**	**005**	**48**					**L07L**	**48**		**113**	D3L	586	95	006	619	Ankyrin repeat protein
		C20L	**103**	**69**	**006**	**64**					**L06L**	**128**		**109**						
		C19L	**259**	**90**	**007**	**109**					**L04L**	**109**		**77**						
					008	**112**								**149**						
HSPV005a	**5051–4779 (91)**										**L03L**	**93**			D4L	672	97	008	672	Ankyrin repeat protein
HSPV005b	**5530–5081 (150)**	**C18L**	**150**	**98**							**L03L**	**93**		**163**	D4L	672	92	008	672	
HSPV005c	**6797–5607 (397)**	**C17L**	**386**	**92**					**003L**	**102**	**L02L**	**416**		**385**	D4L	672	94	008	672	
									004L	**233**										
HSPV006	7419–6961 (153)	**C16L**	**181**	**97**					**189R**	**188**	L01L	147	**002**	**184**	D5L	153	97	009	153	
HSPV007	**8041–7589 (151)**														D6L	219	76	010	215	
HSPV008	9327–8509 (273)														D7L	273	89			BTB/POZ domain protein
HSPV009	**10133–9681 (151)**														D12L	202	96	014	202	Chemokine binding domain protein
HSPV010	10529–10197 (111)														D13L	111	99	015	110	TNFR, CD30-like protein
HSPV011a	**11413–10625 (263)**														D14L	764	95	016	764	Ankyrin repeat protein
HSPV011b	**12197–11334 (288)**														D14L	764	95	016	764	
HSPV011c	**12637–12191 (149)**														D14L	764	97	016	764	
HSPV012	14485–13175 (437)														C1L	437	97	017	435	Ankyrin repeat protein
HSPV013	**15127–14852 (92)**														C2L	178	98	018	171	
HSPV014a	**15498–15220 (93)**														C3L	833	95	019	796	Ankyrin repeat protein
HSPV014b	**16216–15473 (248)**														C3L	833	94	019	796	
HSPV014c	**16975–16367 (203)**														C3L	833	94	019	796	
HSPV014d	**17605–17111 (165)**														C3L	833	89	019	796	
HSPV015a	**17913–17695 (73)**														C4L	170	91	020	170	VACV C7L-like protein
HSPV015b	**18205–17960 (82)**														C4L	170	94	020	170	
HSPV016	18365–18784 (140)	C11R	142	95	009	140	C18R	140	005R	140	005R	140	006	138	C5R	138	88	021	139	Growth factor
HSPV017	19934–18942 (331)	C10L	331	96	010	331	C17L	331	006L	326	007L	331	007	331	C6L	331	96	022	331	
HSPV018	**20448–20765 (106)**				**011**	**181**	**C16R**	**44**	**007R**	**91**	008R	239	008	242	C7R	242	88	023	242	ECTV p28-like host range protein
HSPV019	21707–21330 (126)				013	126	**C15L**	**68**	008L	120	009L	124	009	126	C8L	124	78	024	126	Secreted IL-18 binding protein
HSPV020a	**22038–21769 (90)**				**014**	**237**			**009L**	**90**	**010L**	**90**		**409**	C9L	668	96	025	668	Ankyrin repeat host range protein
HSPV020b	**22479–22054 (142)**				**014**	**237**	**C14L**	**142**	**010L**	**142**	**011L**	**142**		**409**	C9L	668	95	025	668	
HSPV020c	**22999–22571 (143)**				**015**	**137**	**C13L**	**115**	**011L**	**135**	**012L**	**137**		**409**	C9L	668	97	025	668	
HSPV020d	**23508–23278 (77)**				**016**	**77**	**C12L**	**77**	**012L**	**90**	**013L**	**77**		**77**	C9L	668	91	025	668	
HSPV020e	**23745–23533 (71)**				**017**	**71**	**C11L**	**76**	**013L**	**71**	**014L**	**71**		**71**	C9L	668	95	025	668	
HSPV021	24072–23884 (63)				018	60	C10L	59		59		59	010	59	C10L	62	95	026	68	
HSPV022	26149–24248 (634)	C9L	634	99	019	634	C9L	634	**014L**	**109**	016L	634	011	634	C11L	614	69	027	632	Ankyrin repeat protein
									015L	**96**										
									016L	**297**										
HSPV023	26725–26195 (177)	C8L	184	89	020	177	C8L	177	017L	177	019L	177	012	177	C12L	182	88	028	186	
HSPV024	27249–26800 (150)	C7L	150	97	021	150	C7L	150	018L	150	020L	150	013	150	C13L	150	97	029	150	Host range protein
HSPV025	27949–27485 (155)	C6L	151	89	022	151	C6L	151	019L	157	021L	151	014	151	C14L	156	96	030	155	
HSPV026a	**28280–28089 (64)**	C5L	204	98	023	204	C5L	204			022L	204	015	204	C15L	205	98	**031**	**69**	
HSPV026b	**28690–28328 (121)**	C5L	204	98	023	204	C5L	204			022L	204	015	204	C15L	205	96	**032**	**125**	BTB/POZ domain protein
HSPV027a	**29175–28765 (137)**	C4L	316	97	024	316	**C4L**	**255**			023L	316	016	316	C16L	316	97	033	316	
HSPV027b	**29689–29489 (67)**	C4L	316	98	024	316	**C4L**	**255**			023L	316	016	316	C16L	315	93	033	316	
HSPV028	30547–29759 (263)	C3L	263	96	025	263	C3L	263			025L	263	017	263	C17L	259	93	034	263	Complement binding protein
HSPV029	32153–30618 (512)	C2L	512	97	026	512	C2L	512			026L	512	018	512	C18L	512	97	035	512	Kelch-like protein
HSPV030	32891–32223 (223)	C1L	224	97	027	229	C1L	224			027L	224	019	224	C19L	231	96	036	231	
HSPV031	33231–32881 (117)	N1L	117	100	028	117	NIL	117	020L	113	028L	117	020	117	Q1L	117	94	037	117	TLR/IL-1R/TNFR signaling inhibitor
HSPV032	33895–33371 (175)	N2L	175	97	029	175	N2.1L*	175	021L	170	029L	175	021	176	Q2L	175	98	038	177	Alpha-amanitin-sensitive protein

HSPV033a	**34155–33940 (72)**	M1L	472	94	030	472	M1L	472			030L	472	022	472	P1L	474	93	039	473	Ankyrin repeat protein
HSPV033b	**34798–34148 (217)**	M1L	472	97	030	472	M1L	472			030L	472	022	472	P1L	474	97	039	473	
HSPV033c	**35347–34871 (159)**	M1L	472	96	030	472	M1L	472			030L	472	022	472	P1L	474	93	039	473	
HSPV034	35987–35328 (220)	M2L	220	99	031	220	**M2L**	**196**			031L	220	023	220	P2L	163	99	040	220	
HSPV035	36976–36125 (284)	K1L	284	97	032	284	**K1L**	**189**	**022L**	**98**	032L	284	024	284	M1L	284	95	041	284	Ankyrin repeat host range protein
HSPV036	38328–37210 (373)	K2L	369	97	033	369	K2L	369	023L	369	035L	369	025	369	M2L	378	97	042	373	Serpin SPI-3
HSPV037	38644–38381 (88)	K3L	88	96	034	88	K3L	88	024L	88	037L	88	026	88	M3L	88	97	043	88	eIF2α-like PKR inhibitor
HSPV038	39979–38708 (424)	K4L	424	99	035	424	K5L*	424	025L	424	038L	424	027	424	M4L	424	97	044	424	Phospholipase D-like protein
HSPV039	**40845–40159 (229)**	**K5L**	**136**	**95**	**037**	**134**	**K6L**	**134**	**026L**	170	**040L**	**134**		**121**	M5L	276	94	045	276	Monoglyceride lipase
		K6L	**81**	**96**	**038**	**81**					**041L**	**84**		**84**						
HSPV040	40984–41430 (149)	K7R	149	100	039	140	K8R	140	028R	149	042R	140	028	149	M6R	161	96	046	149	
HSPV041	42262–41501 (254)	F1L	226	88	040	226	F1L	226	029L	222	044L	226	029	227	G1L	238	85	048	251	Apoptosis inhibitor
HSPV042	42717–42277 (147)	F2L	147	98	041	147	F2L	147	030L	147	045L	147	030	147	G2L	147	97	049	147	dUTPase
HSPV043	44183–42744 (480)	F3L	480	99	042	480	F3L	480	031L	476	046L	480	031	480	G3L	485	98	050	480	Kelch-like protein
HSPV044	45153–44197 (319)	F4L	319	99	043	319	F4L	319	032L	319	049L	319	032	319	G4L	319	99	051	333	Ribonucleotide reductase small subunit
HSPV045	46150–45188 (321)	F5L	321	96	044	322	F5L	322	**033L**	**97**	050L	321	033	321	G5L	323	95	052	323	
									034L	**218**										
HSPV046	46404–46183 (74)	F6L	74	100	045	74	F6L	74	035L	74	051L	74	034	74	G6L	74	100	053	71	
HSPV047	46674–46423 (84)	F7L	92	90	046	80	F7L	82	036L	80	052L	80	035	84	G7L	80	96	054	81	
HSPV048	47035–46841 (65)	F8L	65	93	047	65	**F8L**	**50**	037L	65	053L	65	036	65	G8L	65	98	055	65	
HSPV049	47733–47098 (212)	F9L	212	99	048	212	F9L	212	038L	212	054L	212	037	212	G9L	212	99	056	212	
HSPV050	49039–47723 (439)	F10L	439	99	049	439	F10L	439	039L	439	056L	439	038	439	G10L	439	99	057	439	Ser/Thr protein kinase
HSPV051	50126–49065 (354)	F11L	354	99	050	348	F11L	354	**040L**	**84**	057L	354	039	354	G11L	354	99	059	354	RhoA-interacting protein
									041L	**100**										
HSPV052	52091–50187 (635)	F12L	635	99	051	635	F12.1L*	635	042L	635	059L	635	040	635	G12L	634	97	060	634	IEV protein
HSPV053	53243–52128 (372)	F13L	372	99	052	372	F13L	372	043L	372	060L	372	041	372	G13L	372	99	061	372	Palmitylated EEV envelope lipase
HSPV054	53482–53264 (73)	F14L	73	98	053	73	F14L	73	044L	73	061L	73	042	73	G14L	73	97	062	73	
HSPV055	54230–53757 (158)	F15L	158	98	054	147	F15L*	158	045L	158	063L	158	043	158	G15L	158	99	064	158	
HSPV056	54932–54240 (231)	F16L	231	98	055	231	F16L	231	046L	231	064L	231	044	231	G16L	231	97	065	231	
HSPV057	54995–55297 (101)	F17R	101	98	056	101	F17R	101	047R	101	065R	101	045	101	G17R	101	99	066	101	DNA binding virion core protein
HSPV058	56736–55300 (479)	E1L	479	99	057	479	E1L	479	048L	479	067L	479	046	479	F1L	479	98	067	479	Poly(A) polymerase large subunit
HSPV059	58946–56736 (737)	E2L	737	99	058	737	E2L	737	049L	737	068L	737	047	737	F2L	737	97	068	737	
HSPV060	59619–59050 (190)	E3L	190	97	059	190	E3L*	190	050L	190	069L	190	048	190	F3L	190	97	069	190	dsRNA binding PKR inhibitor
HSPV061	60453–59677 (259)	E4L	259	99	060	259	E5L	259	051L	259	070L	259	049	259	F4L	259	98	070	261	RNA polymerase subunit RPO30
HSPV062	60530–61522 (331)	E5R	331	97	061	341	**E6R**	**257**	052R	331	071R	341	050	341	F5R	331	94	071	319	
Right-terminal genomic region																				
HSPV146a	**146239–146030 (70)**	**A25L**	**65**	**91**	**145**	**65**	**A27L**	**233**	**136L**	**65**	**185L**	**210**		**233**	A26L	1,279	67	158	1,284	ATI protein
HSPV146b	**146819–146211 (203)**	**A26L**	**322**	**93**	**146**	**154**	**A27L**	**233**			**185L**	**210**		**233**	A26L	1,279	94	158	1,284	
HSPV146c	**147691–147167 (175)**				**147**	**227**	**A29L**	**230**			**187L**	**227**		**227**	A26L	1,279	94	158	1,284	
HSPV146d	**149828–147654 (725)**				**148**	**725**	**A31L**	**725**			**189L**	**725**		**725**	A26L	1,279	97	158	1,284	
HSPV147	151378–149876 (501)	**A26L**	**322**	**98**	149	500	**A33L**	**168**	**137L**	**230**	191L	502	134	500	A27L	518	94	**159**	**192**	IMV ATI-like protein P4c
							A34L	**71**										**161**	**260**	
							A35L	**229**												
HSPV148	151761–151432 (110)	A27L	110	99	150	110	A36L	110	138L	110	192L	110	135	110	A28L	110	99	162	110	IMV membrane protein
HSPV149	152202–151765 (146)	A28L	146	99	151	146	A37L*	146	139L	146	193L	146	136	146	A29L	146	99	163	146	IMV membrane protein
HSPV150	153120–152206 (305)	A29L	305	99	152	305	A38L	305	140L	305	195L	305	137	305	A30L	305	98	164	305	RNA polymerase subunit RPO35
HSPV151	153316–153086 (77)	A30L	77	100	153	77	A39L	77	141L	77	196L	77	138	77	A31L	77	100	165	76	Virion core protein
HSPV152	153476–153874 (133)	**A31R**	**124**	**90**	**154**	**124**	A40R	141	**142R**	**125**	197R	127	**139**	**124**	A32R	145	90	166	140	
HSPV153	**154656–153847 (270)**	A32L	300	99	**155**	**270**	A41L	327	**143L**	**269**	**199L**	**270**	140	300	A33L	300	99	167	311	ATPase, DNA packaging
HSPV154	154774–155328 (185)	A33R	185	100	156	185	A42R*	185	144R	185	200R	185	141	185	A34R	185	98	168	187	EEV envelope protein
HSPV155	155355–155858 (168)	A34R	168	99	157	168	A44R	168	145R	168	201R	168	142	168	A35R	168	97	169	168	EEV envelope protein
HSPV156	155905–156432 (176)	A35R	176	98	158	176	A45R	176	146R	176	202R	176	143	177	A36R	176	98	171	176	
HSPV157	156502–157170 (223)	A36R	221	99	159	221	A46R	221	147R	208	204R	221	144	224	A37R	223	98	172	224	IEV protein
HSPV158	157237–158025 (263)	A37R	263	99	160	263	A47R	268	148R	263	205R	263	145	263	A38R	268	95	173	263	
HSPV159	158139–158324 (62)		64		161	62		64		57	207R	62		**56**	A39R	64	95	174	63	
HSPV160	159157–158327 (277)	A38L	277	95	162	277	Unknown	277	149L	277	208L	277	146	277	A40L	277	97	175	277	CD47-like membrane glycoprotein
HSPV161	**159173–160150 (326)**	A39R	403	93	**163**	**295**	**A49R**	**228**	**150R**	**83**	209R	403	147	401	A41R	402	94	176	409	Semaphorin-like protein
					164	**142**	**A50R**	**142**	**151R**	**210**										
HSPV162	160402–160896 (165)	A40R	168	97	165	159	A51R	159	152R	168	211R	159	148	159	A42R	166	95	177	160	C-type lectin-like membrane protein

Continued on following page

TABLE 1—*Continued*

HSPV ORF	Position (length[b])	VACV[c]										RPXV[d]		CPXV[e]					Putative function/similarity[h]
		CPN			WR		Tian		MVA		mO			GRI			BRI		
		ORF	Length	% Id[f]	ORF	Length	ORF[g]	Length	ORF	Length	ORF	Length	ORF	Length	ORF	Length	% Id[f]	ORF	Length	
HSPV163	161656–161000 (219)	A41L	219	97	166	219	A52L	219	153L	219	212L	219	149	219	A43L	219	97	178	218	Secreted immunomodulatory protein
HSPV164	161827–162225 (133)	A42R	133	97	167	133	A53R	133	154R	128	213R	133	150	133	A44R	133	100	179	133	Profilin-like protein
HSPV165	162266–162850 (195)	A43R	194	93	168	194	A54R	194	155R	190	214R	194	151	194	A45R	196	95	180	194	
HSPV166	164226–163189 (346)	A44L	346	98	170	346	A55L	346	157L	346	216L	346	153	346	A47L	346	98	182	346	Hydroxysteroid dehydrogenase
HSPV167	164273–164647 (125)	A45R	125	99	171	125	A56R	125	158R	121	217R	125	154	125	A48R	125	98	183	125	Superoxide dismutase-like protein
HSPV168	164640–165359 (240)	**A46R**	**214**	**100**	172	240	**A57R**	**210**	159R	240	218R	240	155	240	A49R	240	97	184	242	TLR/IL-1R signaling inhibitor
HSPV169	166183–165452 (244)	A47L	244	99	173	252	A58L	252	160L	238	220L	252	156	244	A50L	244	97	185	244	
HSPV170	**166282–166893 (204)**	**A48R**	**204**	**100**	174	227	**A59R**	**204**	**161R**	**204**	**221R**	**204**	**157**	**204**	A51R	227	99	186	227	Thymidylate kinase
HSPV171	166944–167429 (162)	A49R	162	96	175	162	A60R	162	162R	162	222R	162	158	162	A52R	162	95	187	162	
HSPV172	167464–169119 (552)	A50R	552	98	176	552	A61R	552	163R	552	223R	552	159	552	A53R	552	97	188	554	DNA ligase
HSPV173a	**169175–169414 (80)**	A51R	334	88	177	334	A62R*	334	164R	310	**226R**	**73**		**84**	A54R	334	92	189	334	
HSPV173b	**169359–170168 (270)**	A51R	334	96	177	334	A62R	334	164R	310	**227R**	**266**		**126**	A54R	334	96	189	334	
														120						
HSPV174	170241–170810 (190)	A52R	190	98	178	190	A63R*	**190**			228R	190	160	190	A55R	190	96	190	190	TLR/IL-1R signaling inhibitor
HSPV175	**171134–171439 (102)**	**A53R**	**108**	**91**	**179**	**103**	*	**137**			229R	186	**161**	**102**	A56R	186	88	191	186	TNFR, CrmC
		AORFT	**81**	**100**			*	**103**												
HSPV176	171969–173660 (564)	A55R	564	99	180	564	A65R*	564			232R	564	162	564	A57R	564	97	193	563	Kelch-like protein
HSPV177	173713–174654 (314)	A56R	315	98	181	314	A66R	315	165R	315	233R	310	**163**	**206**	A58R	314	94	194	297	EEV hemagglutinin
HSPV178a	**174675–174860 (62)**	**A57R**	**151**	**93**	**182**	**151**	**A67R**	**151**			**234R**	**37**	**164**	**151**	A59R	197	96	195	197	Guanylate kinase
HSPV178b	**174975–175265 (97)**	**A57R**	**151**	**100**	**182**	**151**	**A67R**	**151**	**166R**	**97**	**235R**	**151**	**164**	**151**	A59R	197	95	195	197	
HSPV179	175419–176318 (300)	B1R	300	99	183	300	B1R	300	167R	300	236R	300	165	300	B1R	300	99	196	299	Ser/Thr protein kinase, DNA replication
HSPV180a	**176412–177068 (219)**	**B2R**	**219**	**96**	**184**	**219**	**B2R**	**219**	**168R**	**96**	**238R**	**219**		**219**	B2R	503	93	197	505	
									169R	**143**										
HSPV180b	**177107–177478 (124)**	**B3R**	**124**	**96**	**185**	**167**	**B3R**	**124**	**170R**	**179**	**240R**	**124**		**124**	B2R	503	91	197	505	Schlafen-like protein
HSPV181	178138–179811 (558)	B4R	558	98	186	558	B4R	558	**171R**	**177**	242R	558	166	558	B3R	558	95	198	558	Ankyrin repeat protein
									172R	**409**										
HSPV182	179917–180867 (317)	B5R	317	97	187	317	B5R	317	173R	317	243R	317	167	317	B4R	317	96	199	317	EEV host range protein
HSPV183a	**180953–181285 (111)**	B6R	173	99	188	173	B6R	173	174R	173	244R	173	168	173	B5R	183	93	200	179	
HSPV183b	**181288–181482 (65)**	B6R	173	100	188	173	B6R	173	174R	173	244R	173	168	173	B5R	183	93	200	179	
HSPV184	181523–182068 (182)	B7R	182	100	189	182	B7R	182	175R	177	246R	182	169	182	B6R	182	97	201	181	Chemokine binding domain
HSPV185	182149–182964 (272)	B8R	272	97	190	272	B8R	272	**176R**	**226**	247R	272	170	272	B7R	271	97	202	266	IFN-γ receptor
HSPV186	**183054–183284 (77)**	**B9R**	**77**	**100**	**191**	**77**	**B9R**	**77**	**177R**	**72**	**248R**	**77**		**61**	B8R	221	97	203	225	MYXV M-T4-like protein
HSPV187	**183384–183746 (121)**	**B10R**	**166**	**99**	**192**	**166**	**B10R**	**166**	**178R**	**158**	**249R**	**166**	**172**	**166**	B9R	501	94	204	501	Kelch-like protein
HSPV188	**183821–184054 (78)**	**B11R**	**88**	**96**	**193**	**72**	**B11R**	**76**	**179R**	**74**	**250R**	**72**	**173**	**72**	B10R	105	96	205	90	
HSPV189	184124–184972 (283)	B12R	283	98	194	283	B12R	283	180R	283	251R	283	174	283	B11R	283	98	206	285	Ser/Thr protein kinase
HSPV190	185074–186108 (345)	**B14R**	**222**	**97**	195	345	**B14R**	**222**	**181R**	**116**	**253R**	**222**	175	345	B12R	345	93	207	341	Serpin, SPI-2
									182R	**222**										
HSPV191	186186–186632 (149)	B15R	149	97	196	149	B15R	149	183R	143	254R	149	176	149	B13R	149	98	208	149	
HSPV192	186736–187713 (326)	**B16R**	**290**	**97**	197	326	**B16R**	**290**	184R	326	255R	326		**207**	B14R	326	95	209	326	IL-1 receptor
														134						
HSPV193	188784–187765 (340)	B17L	340	97	198	340	B17L	340	185L	340	257L	340	177	340	B15L	340	96	210	340	
HSPV194	188921–190642 (574)	B18R	574	98	199	574	B18R	574	186R	574	**258R**	**413**	178	574	B16R	574	95	211	574	Ankyrin repeat protein
HSPV195	190711–191775 (355)	B19R	353	98	200	351	B19R	353	**187R**	**234**			179	351	B17R	351	91	212	366	IFN-α/β binding protein
HSPV196	191850–194222 (791)	**B20R**	**127**	**96**	**202**	**53**	**B20R***	**613**					180	791	B18R	795	94	213	800	Ankyrin repeat protein
					203	**309**														
HSPV197	194331–195971 (547)				**204**	**134**									B19R	557	94	215	557	Kelch-like protein
HSPV198	196272–197342 (357)	C12L	353	97	205	353	C19L	353			004L	353	005	357	B20R	375	95	217	372	Serpin, SPI-1
HSPV199	197515–198090 (192)	**C13L**	**65**	**90**	206	190					003L	190	004	192	B21R	190	94	218	198	Chemokine binding domain protein
		C14L	**82**	**98**																
HSPV200	198347–204106 (1,920)								**188R**	**70**					B22R	1,033	97	219	1,019	VARV B22R-like protein
HSPV201a	**204447–204806 (120)**	**B21R**	**91**	**96**							**002L**	**89**	**003**	**91**	K1R	581	96	220	579	Ankyrin repeat protein
HSPV201b	**204961–205161 (67)**								**189R**	**188**					K1R	581	90	220	579	

HSPV202 205215–205673 (153)	**B22R**	**181**	**97**			**189R**	**188**	**L01L**	**147**	**002**	**184**	I1R	153	97	222	153		
HSPV203a205837–207027 (397)	**B23R**	**386**	**92**			**190R**	**233**	**L02L**	**416**		**385**	I2R	672	94	223	672	Ankyrin repeat protein	
						191R	**102**											
HSPV203b207104–207553 (150)	**B24R**	**150**	**98**					**L03L**	**98**		**163**	I2R	672	92	223	672		
HSPV203c207583–207855 (91)								**L03L**	**93**			I2R	672	97	223	672		
HSPV204 208064–209824 (587)	**B25R**	**259**	**90**	**211**	**112**			**L04L**	**100**		**140**	I3R	586	95	225	619	Ankyrin repeat protein	
	B26R	**103**	**69**	**212**	**109**			**L06L**	**128**		**77**							
	B27R	**113**	**100**	**213**	**64**			**L07L**	**48**		**109**							
				214	**48**						**113**							
HSPV205 209912–210958 (349)	**B28R**	**122**	**91**	**215**	**122**		**192R**	**176**	**L08L**	**122**	**122**	I4R	351	95	226	355	TNFR II-like protein, CrmB	
				217	**61**				**L09L**	**34**	**63**							
HSPV206 211087–211830 (248)	B29R	244	86	218	244	B23R	244	**193R**	**136**	L10L	258	001	258	I5R	255	87	227	246	Chemokine binding protein
HSPV207 211946–212161 (72)																	229	64	

[a] Boldface indicates ORFs >10% different in length from intact orthologues from CPXV GRI-90 or BRI. Names of ORF homologues have been abbreviated here for simplicity and lack the following prefixes for the following viruses: VACV WR, WR; T, Tian Tan; MVA, MVA; m0LTR, ORFs in the m0 long terminal repeat indicated here with prefix L; m0, unique m0 ORFs; RPXV, RPXV; CPXV, BRI.

[b] All lengths are in amino acids.

[c] VACV strains (accession numbers): CPN (M35027); WR (AY243312); Tian, Tian Tan (AF095689); MVA (U94848); m0, LC16m0 (AY678277). Larger ORFs matching multiple HSPV ORFs are VACV CPN ORFs C5L, C4L, M1L, A26L, A51R, A57R, and B6R; VACV WR ORFs 014, 023, 024, 030, 177, 182, and 188; VACV Tian Tan ORFs C5L, C4L, M1L, A27L, A62R, A67R, and B6R; VACV MVA ORFs 164R, 174R, and 189R; and VACV m0 ORFs L03L, 022L, 023L, 030L, 185L, and 244R.

[d] RPXV strain Utrecht. RPXV ORF lengths lacking an ORF designation indicate ORFs lacking translation products annotated in sequence AY484669. Larger ORFs matching multiple HSPV ORFs are the 409-amino-acid-long ORF, ORF 015, ORF 016, ORF 022, the 233-amino-acid-long ORF, ORF 164, and ORF 168.

[e] CPXV strains (accession numbers): GRI, GRI-90 (X94355); BRI (AF482758). Larger ORFs matching multiple HSPV ORFs are GRI-90 ORFs D4L, D14L, C3L, C4L, C9L, C15L, C16L, P1L, A26L, A54R, A59R, B2R, B5R, K1R, and 12R and BRI ORFs 008, 016, 019, 020, 025, 033, 039, 158, 189, 195, 197, 200, 220, and 223.

[f] % Id, percent amino acid identity in local BLAST match.

[g] Asterisks indicate ORFs resequenced/reannotated by Upton et al. (92) as present, intact, or fused to a subsequent ORF in the Tian Tan genome.

[h] Abbreviations: IL-18, interleukin-18; TLR, Toll-like receptor; PKR, double-stranded RNA-dependent protein kinase; IEV, intracellular enveloped virion; IFN-γ, gamma interferon; MYXV, myxoma virus; dsRNA, double-stranded RNA.

respectively), indicating that HSPV is similar to other OPVs in these functions (59) (Table 2).

HSPV terminal genomic regions were similar to other OPVs in that they contained a homologous subset of the sequence and intact ORFs present in various strains of CPXV, viruses found to contain a relatively complete OPV genotype and thus thought to be viruses from which other OPV lineages are derived following gene fragmentation and loss (Table 1; Fig. 1) (75, 79). Many of these ORFs have been characterized in other OPVs as affecting viral virulence, host range, and modification of host responses, including apoptosis and innate and adaptive immune mechanisms (59, 60, 82). However, the specific subset of genes present in HSPV was unique relative to other OPVs, containing terminal genomic sequences not characteristic of currently known OPVs and including approximately 1.4 kb of sequence found only in CPXV (located between positions 15453 and 16985) (Fig. 1).

Phylogenetic analysis. Phylogenetic analysis of OPV genomic regions, including the highly conserved central region and parts of the more variable terminal regions, indicated that HSPV is closely related to sequenced strains of VACV and RPXV, falling very close to or within this VACV subgroup (referred to here as VACV-like viruses) relative to other OPVs (Fig. 2). These results are consistent with those obtained previously for OPVs, with VACV-like viruses closely related to each other compared to other OPV species, and they indicated that HSPV is a VACV-like virus (21, 38, 51). As a VACV-like virus, HSPV also shares a closer relationship with CPXV strain GRI-90 than with CPXV strain Brighton Red (BRI), consistent with previous OPV phylogenetic analyses and indicating the distinct nature of CPXV species despite the relative conservation in gene content (Fig. 1 and 2) (21, 38, 51). Similarly, a close relationship was observed between HSPV and VACV using concatenated right terminal OPV gene sequences used previously for OPV phylogenetic analysis (HSPV177, HSPV179, HSPV182, and HSPV191; data not shown) (38). These results indicate that HSPV and VACV are very similar phylogenetically and share a relatively recent common ancestor. Notably, HSPV had a slightly greater estimated distance to VACV-like isolates than they demonstrated to each other, with HSPV tending to fall outside the rest of the VACV-like cluster (Fig. 2). These data suggested that, while very closely related, HSPV is phylogenetically distinct from other characterized VACV-like viruses.

Comparison of HSPV with VACV-like viruses. Given the close phylogenetic relationship between HSPV and VACV-like viruses, HSPV ORFs were compared to VACV-like homologues in the more variable terminal genomic regions which tend to contain genes dispensable for basic replicative processes but important for specific virus-host interaction and aspects of virulence and host range (Fig. 1; Table 1). While HSPV maintained a high level of amino acid identity where homologous terminal region ORFs were present (average of 95% amino acid identity to CPN), we focused here on comparison of HSPV and VACV in genes likely fragmented relative to CPXV and other OPVs. Overall, these differences often involved genes that are members of multigene families and/or homologues of genes shown or thought to affect OPV virulence or host range, among them those that code for ankyrin repeat proteins, kelch-like proteins, and tumor necrosis factor recep-

TABLE 2. HSPV ORFs in central genomic regions compared to orthologues annotated in VACV CPN[a]

HSPV ORF	Position (length[c])	VACV CPN		Putative function/similarity
		ORF	Length	
HSPV063	61662–63362 (567)	E6R	567	
HSPV064	63447–63944 (166)	E7R	166	
HSPV065	64072–64890 (273)	E8R	273	Virion core protein
HSPV066	67919–64902 (1,006)	E9L	1,006	DNA polymerase
HSPV067	67951–68235 (95)	E10R	96	IMV redox protein
HSPV068	68622–68236 (129)	E11L	129	Virion core protein
HSPV069	70609–68612 (666)	O1L	666	
HSPV070	70983–70660 (108)	O2L	108	Glutaredoxin
HSPV071	72067–71132 (312)	I1L	312	DNA binding virion core protein
HSPV072	72304–72077 (76)	I2L	73	
HSPV073	73114–72308 (269)	I3L	269	DNA binding phosphoprotein
HSPV074a	**73439–73200 (80)**	I4L[b]	771	Ribonucleotide reductase large subunit
HSPV074b	**74885–73566 (440)**	I4L	771	
HSPV074c	**75213–74842 (124)**	I4L	771	
HSPV074d	**75503–75216 (96)**	I4L	771	
HSPV075	75770–75534 (79)	I5L	79	IMV membrane protein
HSPV076	76937–75792 (382)	I6L	382	Telomere binding protein
HSPV077	78201–76933 (423)	I7L	423	Virion core proteinase
HSPV078	78207–80234 (676)	I8R	676	RNA helicase NPH-II
HSPV079	82016–80244 (591)	G1L	591	Metalloprotease
HSPV080	82342–83001 (220)	G3L	220	
HSPV081	82348–82016 (111)	G2R	111	Transcriptional elongation factor
HSPV082	83348–82977 (124)	G4L	124	Glutaredoxin 2
HSPV083	83351–84652 (434)	G5R	434	Virion core protein
HSPV084	84663–84851 (63)	G5.5R	63	RNA polymerase subunit RPO7
HSPV085	84856–85350 (165)	G6R	166	
HSPV086	86433–85321 (371)	G7L	371	Virion core protein
HSPV087	86464–87243 (260)	G8R	260	Late transcription factor VLTF-1
HSPV088	87266–88285 (340)	G9R	340	Myristylated protein
HSPV089	88289–89038 (250)	L1R	250	Myristylated IMV envelope protein
HSPV090	89073–89333 (87)	L2R	87	
HSPV091	90378–89329 (350)	L3L	350	
HSPV092	90403–91155 (251)	L4R	251	DNA binding virion core protein
HSPV093	91168–91551 (128)	L5R	128	IMV membrane protein
HSPV094	91511–91969 (153)	J1R	153	IMV membrane protein
HSPV095	91988–92518 (177)	J2R	177	Thymidine kinase
HSPV096	92587–93585 (333)	J3R	333	Poly(A) polymerase small subunit
HSPV097	93503–94057 (185)	J4R	185	RNA polymerase subunit RPO22
HSPV098	94585–94187 (133)	J5L	133	
HSPV099	94692–98549 (1,286)	J6R	1,286	RNA polymerase subunit RPO147
HSPV100	99064–98552 (171)	H1L	171	Tyr/Ser protein phosphatase
HSPV101	99078–99644 (189)	H2R	189	IMV membrane protein
HSPV102	100624–99653 (324)	H3L	324	IMV envelope protein
HSPV103	103012–100628 (795)	H4L	795	RNA polymerase-associated protein
HSPV104	103198–103830 (211)	H5R	203	Late transcription factor VLTF-4
HSPV105	103834–104775 (314)	H6R	314	DNA topoisomerase IB
HSPV106	104815–105252 (146)	H7R	146	
HSPV107	105299–107830 (844)	D1R	844	mRNA capping enzyme large subunit
HSPV108	108225–108935 (237)	D3R	237	Virion core protein
HSPV109	108232–107795 (146)	D2L	146	Virion core protein
HSPV110	108938–109591 (218)	D4R	218	Uracil DNA glycosylase
HSPV111	109626–111980 (785)	D5R	785	NTPase, DNA replication
HSPV112	112024–113934 (637)	D6R	637	Early transcription factor small subunit
HSPV113	113964–114446 (161)	D7R	161	RNA polymerase subunit RPO18
HSPV114	115326–114415 (304)	D8L	304	IMV membrane protein, cell binding
HSPV115	115368–116006 (213)	D9R	213	MutT motif
HSPV116	116006–116749 (248)	D10R	248	MutT motif
HSPV117	118648–116756 (631)	D11L	631	NPH-I, transcription termination factor
HSPV118	119546–118686 (287)	D12L	287	mRNA capping enzyme small subunit
HSPV119	121232–119580 (551)	D13L	551	Rifampin resistance protein
HSPV120	121708–121259 (150)	A1L	150	Late transcription factor VLTF-2
HSPV121	122403–121732 (224)	A2L	224	Late transcription factor VLTF-3
HSPV122	122630–122403 (76)	A2.5L	76	Virion redox protein
HSPV123	124579–122648 (644)	A3L	644	Virion core protein P4b
HSPV124	125477–124635 (281)	A4L	281	Virion core protein
HSPV125	125515–126006 (164)	A5R	164	RNA polymerase subunit RPO19

Continued on facing page

TABLE 2—*Continued*

HSPV ORF	Position (length[c])	VACV CPN		Putative function/similarity
		ORF	Length	
HSPV126	127124–126009 (372)	A6L	372	
HSPV127	129280–127151 (710)	A7L	710	Early transcription factor large subunit
HSPV128	129334–130197 (288)	A8R	288	Intermediate transcription factor VITF-3
HSPV129	130501–130196 (102)	A9L	99	IMV membrane protein
HSPV130	133177–130505 (891)	A10L	891	Virion core protein P4a
HSPV131	133192–134145 (318)	A11R	318	Nonstructural protein
HSPV132	134725–134153 (191)	A12L	192	Virion core protein
HSPV133	134961–134752 (70)	A13L	70	IMV membrane protein
HSPV134	135341–135072 (90)	A14L	90	IMV membrane protein
HSPV135	135519–135361 (53)	A14.5L	53	IMV membrane protein
HSPV136	135793–135512 (94)	A15L	94	Virion core protein
HSPV137	136913–135780 (378)	A16L	378	Myristylated IMV membrane protein
HSPV138	137527–136919 (203)	A17L	203	Phosphorylated IMV membrane protein
HSPV139	137542–139020 (493)	A18R	493	DNA helicase, transcriptional elongation
HSPV140	139237–139007 (77)	A19L	77	
HSPV141	139590–140867 (426)	A21L	426	DNA polymerase processivity factor
HSPV142	139591–139241 (117)	A20R	117	IMV membrane protein
HSPV143	140833–141360 (176)	A22R	176	Holliday junction resolvase
HSPV144	141383–142528 (382)	A23R	382	Intermediate transcription factor VITF-3
HSPV145	142528–146019 (1,164)	A24R	1,164	RNA polymerase subunit RPO132

[a] Boldface indicates ORFs >10% different in length from intact orthologues from CPXV GRI-90 or Brighton Red.
[b] I4L is a larger ORF matching multiple HSPV ORFs.
[c] Lengths are in amino acids.

tors (TNFRs) (4, 45, 77). While terminal-region genotypes vary both among OPVs and between known VACV-like viruses, HSPV contained features similar to known VACV-like viruses relative to other OPVs and features that were quite novel (Table 1; Fig. 1).

HSPV genetic features similar to VACV. Genotypic similarity between HSPV and other VACV-like viruses included a number of genes that were fragmented relative to CPXV and occasionally relative to other OPVs. These genes included several which were fragmented or arranged in a similar fashion between HSPV and VACV-like viruses, commensurate with their close phylogenetic relationship (Table 1; Fig. 2). HSPV genes sharing similar ORF fragments with those in certain VACVs include HSPV005/HSPV203 and HSPV020, genes encoding ankyrin proteins and fragmented or missing in most OPVs (Fig. 1A). HSPV005b/HSPV203b in the ITR represents the same fragment of GRI-90 D4L/I2R as CPN C18L/B24R. HSPV020a to -e and similar ORFs in VACV are homologous fragments of CPXV CHOhr, a gene which enables replication of VACV in the normally nonpermissive CHO cell line and affects eukaryotic initiation factor 2α (eIF2α) phosphorylation in HeLa cells (41, 85). Other HSPV ORFs with similar VACV fragments included HSPV146d, HSPV180, and HSPV186. HSPV146d encodes the same 725-amino-acid amino-terminal fragment of the A-type inclusion (ATI) protein present in several VACV-like viruses and expressed in some as a soluble 94-kDa protein (26). HSPV186 is a VACV-like ORF fragment homologous to the amino-terminal region of the OPV homologue of myxoma virus M-T4, a protein important for virulence and infection of lymphocytes by myxoma virus (12). The HSPV186 homologue is expressed in VACV strain Western Reserve (WR); however, deletion mutants were not affected for viral growth in vitro or virulence in mice (68). While amino-terminal M-T4-like fragments are also present in certain strains of MPXV (22, 52), the large nucleotide deletion affecting HSPV186 was characteristic of VACV (Fig. 1C). Also characteristic of VACV are homologues of HSPV180a and HSPV180b (CPN B2R and B3R, respectively), apparent fragments of a larger ORF intact in all OPV species other than VACV and VARV and previously annotated as similar to cellular Schlafen, a family of variably sized proteins with the prototypical 337-amino-acid murine Schlafen 1 recently shown to target cyclin D1 pathways during induction of cellular mid-G_1 cell cycle arrest (15, 39). Notably, HSPV180a and HSPV180b revealed the bipartite nature of the larger OPV homologue, with Schlafen similarity present in the HSPV180b-like (carboxyl-terminal) region and the HSPV180a-like (amino-terminal) region sharing similarity with the putative B2R homologue of *Melanoplus sanquinipes* entomopoxvirus (MSV237) and limited similarity with ORFs of unknown function (p26) from nucleopolyhedrosis viruses (data not shown). While maintenance of these two domains as separate ORFs in HSPV and VACV conceivably suggests function, HSPV180b and VACV orthologues lack carboxyl-terminal sequences both present in the intact OPV ORF and similar to the carboxyl terminus of cellular Schlafen. Overall, similar fragmentation patterns between HSPV and VACV potentially represent shared, derived characters.

Several genes fragmented in HSPV were also fragmented in certain VACV-like isolates but intact in others (Table 1). HSPV ORF fragments with intact homologues in certain VACVs included HSPV018, HSPV161, HSPV173a and -b, and HSPV175. HSPV018 is an amino-terminal fragment homologue of the ECTV p28 ubiquitin ligase, a protein critical for ECTV virulence and macrophage host range and having intact homologues in all other OPV species (74, 87) (Fig. 1). While this gene is also fragmented in several VACV strains, intact homologues have been identified in VACV strains IHD-W and

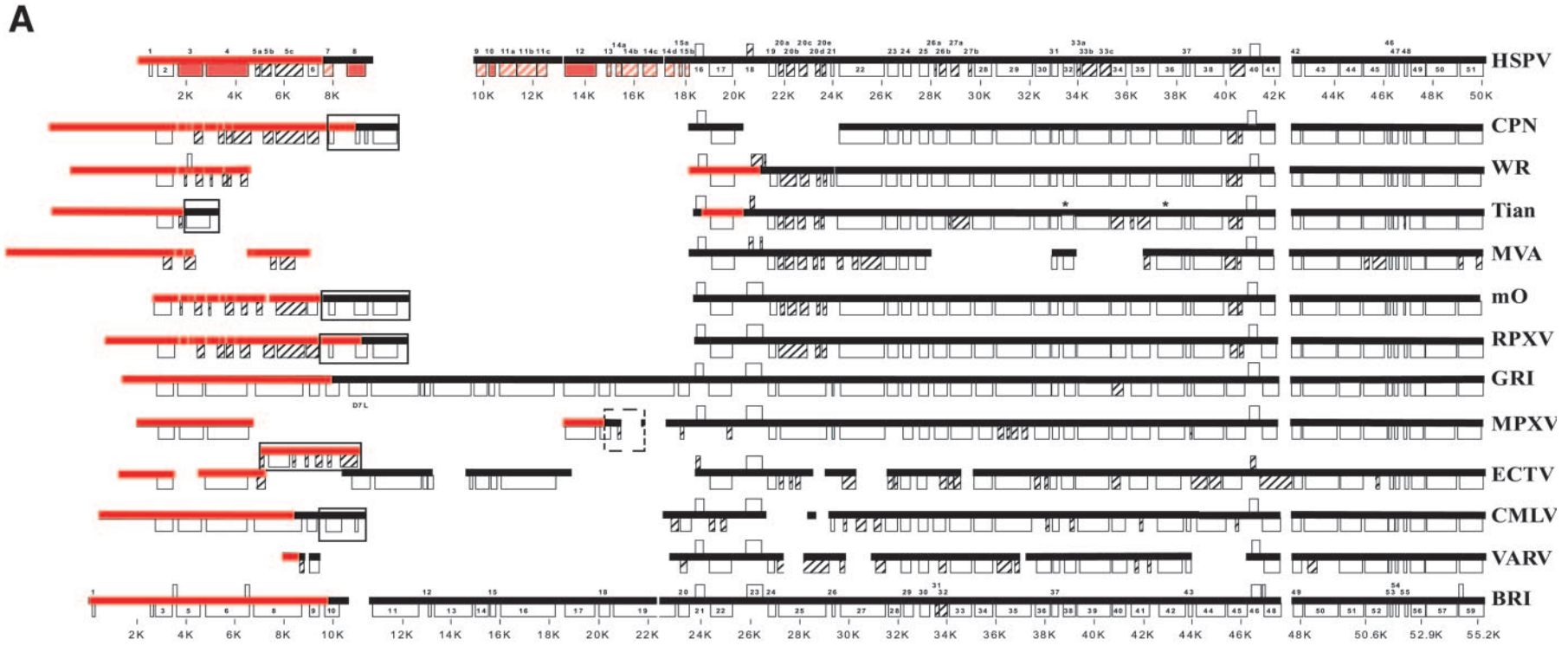

FIG. 1. Schematic comparison of HSPV left (A) and right (B and C) terminal genomic regions to those of other orthopoxviruses. Virus names were abbreviated as follows and correspond to sequences from the following GenBank accession numbers in parentheses: VACV CPN (M35027); VACV WR (AY243312); Tian, VACV Tian Tan (AF095689); VACV MVA (U94848); m0, VACV Lister isolate LC16m0 (AY678277); RPXV, RPXV Utrecht (AY484669); GRI, CPXV GRI-90 (X94355); MPXV, MPXV Zaire-96-I-16 (AF380138); ECTV, ECTV Moscow (AF012825); CMLV, CMLV M-96 (AF438165); VARV, VARV Bangladesh-1975 (L22579); CPXV BRI (AF482758). Heavy lines indicate nucleotide sequences; boxes indicate ORFs matching those annotated in HSPV and those in genomic regions absent in HSPV. ORF names and genomic positions in kilobase pairs (K) are indicated for HSPV and CPXV Brighton Red, as are names of ORFs absent in these two species. Hatching indicates ORFs different in length (>10%) from intact orthologues from CPXV GRI-90 or Brighton Red. Red ORFs indicate HSPV ORFs intact or carried on sequences that are absent relative to VACV-like viruses. Asterisks indicate ORFs resequenced/reannotated by Upton et al. (92) as present or intact in the Tian Tan genome. Large solid-lined boxes indicate sequences matching the global alignment only at the opposite genomic terminus; dashed boxes indicate where sequences located in the opposite genomic terminus match the global alignment. Red lines indicate ITR sequence in each virus and are unaligned on the terminal side of HSPV002/HPSV206. Panel A is presented at a different scale relative to panels B and C.

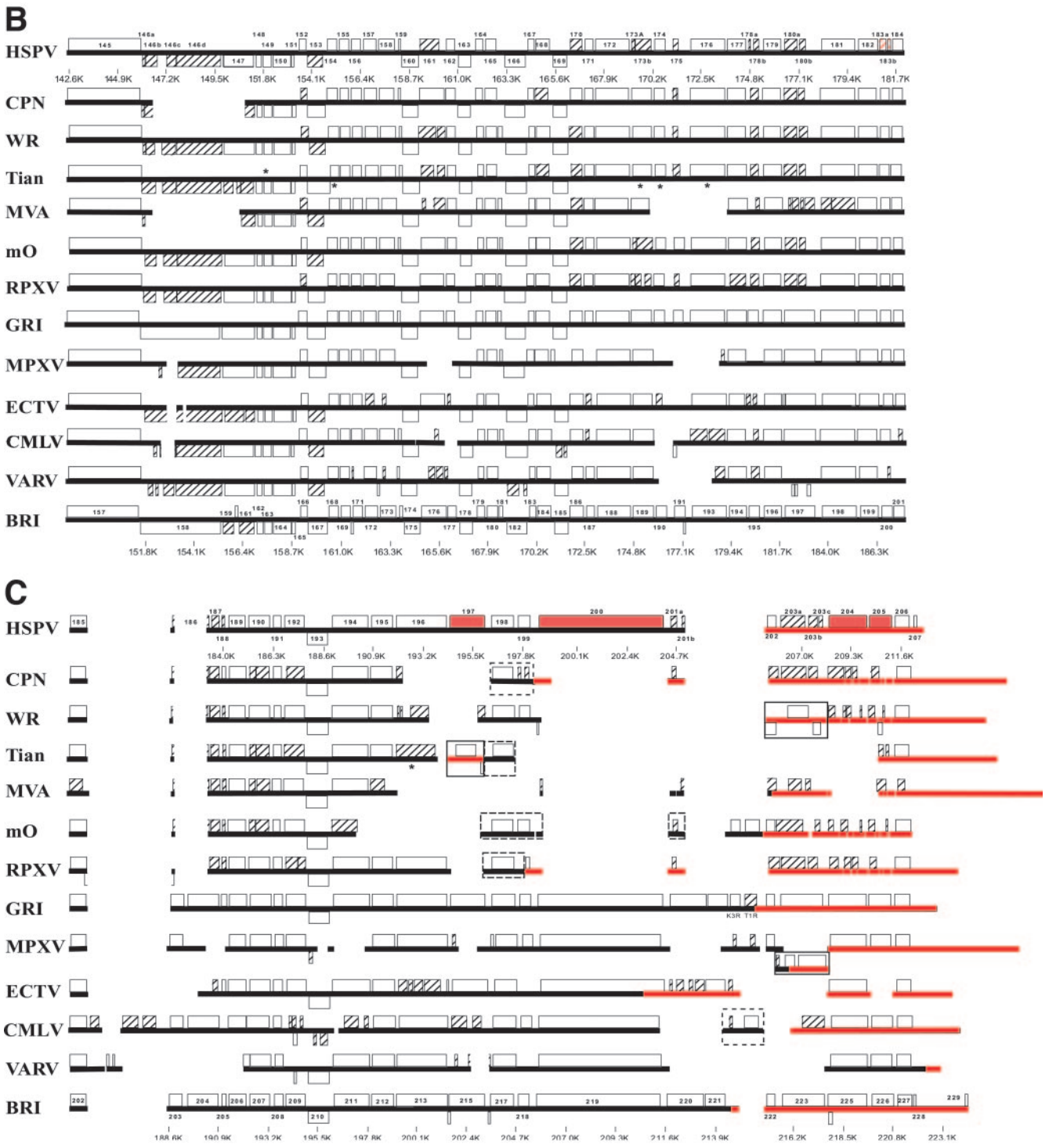

FIG. 1—*Continued.*

Lister and in RPXV (51, 58, 91). Similarly, HSPV173a and -b resembled homologous ORFs in VACV Lister and RPXV and fragments of the CPN A51R gene intact in other VACV strains and all other OPVs. HSPV161 was a homologue of CPN A39R, a secreted semaphorin affecting viral virulence and host inflammatory responses during infection, but, similarly to homologues in WR and other VACV strains, contained a carboxyl-terminal truncation that may predict a nonfunctional product (35). HSPV175, similar to several VACV-like viruses, encoded a truncated copy of the intact CrmC TNFR-like protein encoded by VACV strains Lister, Evans, and USSR (5). HSPV039 and HSPV187 were fragmented genes with homo-

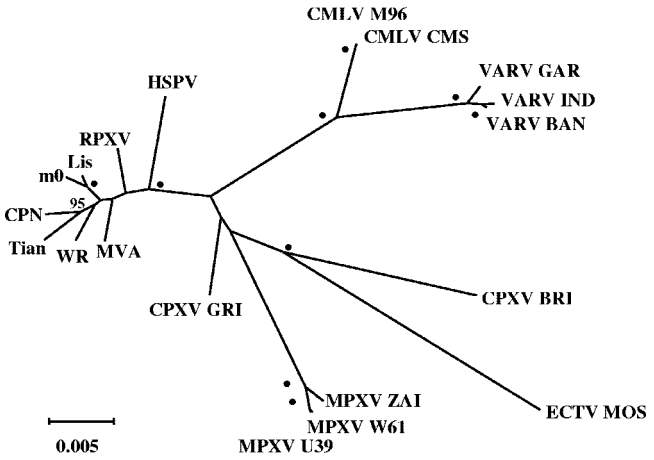

FIG. 2. Phylogenetic analysis of HSPV central genomic regions. Conserved HSPV central genomic nucleotide sequences (positions 26800 to 170171) corresponding to regions used previously for OPV phylogenetic analysis (51) were aligned with homologous OPV sequences using DIALIGN, and gapped regions were realigned with CLUSTAL W and trimmed with Gblocks. The unrooted tree for 124,677 aligned characters was generated using maximum likelihood with general time reversible correction for multiple substitutions, four-category discrete gamma model, estimation for proportion of invariant residues, and 100 bootstrap replicates as implemented in PHYML. Bootstrap values greater than 70 are indicated at appropriate nodes; dots indicate values of 100. Homologous nucleotide sequences from the following viruses and accession numbers were compared: VACV strain CPN, M35027; VACV WR, AY243312; VACV Lister (Elstree) vaccine consensus (Lis), AY678276; VACV Lister-derived LC16m0 (m0), AY678277; VACV Tian Tan (Tian), AF095689; VACV MVA, U94848; RPXV Utrecht (RPXV), AY484669; CPXV strain GRI-90 (X94355); CPXV BRI, AF482758; MPXV strain Zaire-96-I-16 (MPXV ZAI), AF380138; MPXV WRAIR7-61 (MPXV W61), AY603973; MPXV USA_2003_039 (MPXV U39), DQ011157; CMLV strain M-96 (CMLV M96), AF438165; CMLV CMS, AY009089; VARV strain Bangladesh-1975 (VARV BAN), L22579; VARV India-1967 (VARV IND), X69198; VARV Garcia-1966 (VARV GAR); Y16780; ECTV strain Moscow (ECTV MOS), AF012825. The scale indicates estimated distance. Identical topologies at supported nodes were obtained using additional maximum likelihood analyses as implemented in TREE-PUZZLE, using neighbor-joining and maximum parsimony as implemented in PHYLO_WIN and PHYLIP, respectively, and using an unedited alignment (146,439 characters) (data not shown). Similar topologies were also obtained using similar analyses on whole-genomic alignments (data not shown).

logues fragmented in all VACV-like viruses but with VACV-like homologues fragmented in a pattern distinct from those in HSPV. HSPV039 was similar to both CPN K5L and K6L fragments of the OPV monoglyceride lipase-like gene but was much closer in size to the intact CPXV homologue, and HSPV187 was a smaller fragment of the CPXV GRI-90 B9R kelch-like protein. While HSPV175, HSPV039, and HSPV187 homologues were fragmented in both HSPV and most VACV-like viruses, these genes were also disrupted in most other OPVs (Fig. 1).

HSPV also contained intact genes whose homologues were intact in certain VACV-like viruses but disrupted in others, similar to genes recently described in the RPXV genome (Table 1) (51). HSPV002/HSPV206 in the ITR encoded the OPV 35-kDa secreted chemokine binding protein and, similarly to the functional, full-length protein expressed by VACV Lister and other OPVs, lacked the amino-terminal mutation preventing expression of functional protein in CPN, WR, and VACV strain Tian Tan (6). HSPV147 was an intact copy of the gene encoding P4c, a protein involved with direction of IMV to insoluble ATIs but with homologues fragmented or absent in CPN, Tian Tan, and modified vaccinia Ankara (MVA). HSPV190 was only the third intact VACV-like orthologue of the serine proteinase inhibitor (serpin) 2 (SPI-2) to be identified, and HSPV198 was an intact orthologue of the SPI-1 gene intact in most VACV-like viruses but transposed to the opposite terminus in RPXV and VACV CPN, Tian Tan, and Lister and absent in MVA (Fig. 1C). Intact SPI-1 and SPI-2 exhibit antiapoptotic and/or anti-inflammatory activity through inhibition of caspases and have been shown to affect viral virulence and/or host range (48, 59, 82, 87). HSPV196 encodes an intact ankyrin repeat protein truncated by deletion in all VACV-like viruses except RPXV, where the homologue was recently identified as unique among VACV-like viruses in that the entire nucleotide region encompassing the gene was present (51). Similarly, HSPV199 encodes an intact homologue of the BRI CPXV218 chemokine binding protein, with intact homologues also encoded in the right terminus of WR and in the left terminus of VACV Lister and RPXV (Fig. 1) (7). Overall, different fragmentation patterns or gene loss between HSPV genes and VACV homologues may indicate sequence divergence after functional gene loss or, alternatively, could conceivably reflect independent loss of gene function in different VACV-like lineages during convergent adaptation toward similar virulence or host range phenotypes. Gene loss near ITR boundaries may reflect loss during terminal transposition events (47, 61). These phenomena would help explain gene fragmentation that is variable both within the VACV-like lineage and between OPV species.

HSPV genetic features distinct from VACV. Despite sharing specific genomic and genotypic features with some or all known VACV-like viruses within the range of VACV-like genotypic heterogeneity, HSPV contained many features that were unique. These included genes uniquely intact in HSPV but for which homologous nucleotide sequence was present in other VACVs, and they included HSPV genes, both intact and fragmented, that were associated with nucleotide sequences completely novel among VACV-like viruses, resulting in terminal genomic regions encoding additional proteins and protein fragments resembling those in CPXV (94% average amino acid identity to CPXV GRI-90 orthologues) (Fig. 1; Table 1). Finally, HSPV demonstrated unique fragmentation of several genes, including those that were intact in all or most other known VACV-like viruses.

HSPV contained in the ITRs intact genes that are fragmented or absent in all other VACVs (Table 1). HSPV003/HSPV205 is an intact homologue of the secreted CPXV Brighton Red CrmB TNFR II-like protein (CPXV GRI-90 D2L/I2R), a protein which interacts with and inhibits TNF and lymphotoxin alpha and whose orthologue in VARV has been recently shown to contain a novel carboxyl-terminal chemokine binding domain also present and active in several other OPV proteins (4, 7, 42). HSPV004/HSPV204 encodes an intact homologue of the ankyrin repeat protein encoded by CPXV GRI D3L/I3R and intact homologues in MPXV, ECTV, and CMLV (Fig. 1).

HSPV contains approximately 17 kbp of sequence in three distinct genomic regions (positions 7527 to 18195 in the left terminal region and 194379 to 195517 and 198775 to 204285 in the right terminal region) absent in known VACV-like viruses but homologous to sequences in sequenced strains of CPXV and other OPVs (Fig. 1). HSPV also contains approximately 1.4 kbp of sequence absent not only in VACV but also in all known OPVs except CPXV. For this region, located between positions 15453 and 16985, only MPXV contains a fragment (approximately 75 bp) of homologous sequence. Notably, sequences near this region reflect ITR and/or terminal translocations in several OPVs (Fig. 1), and repetitive sequence near this locus in ECTV has been suggested to be a dynamic genomic region (21). Conceivably, the presence of this 1.4-kbp sequence in HSPV is consistent with retention of adjacent and relatively significant amounts of CPXV-like sequence in this left terminal region relative to other OPVs (Fig. 1).

HSPV sequence in the left terminal region absent in other VACV-like viruses corresponds to the D7L loci of CPXV GRI-90 and the CPXV014 to CPXV020 region of CPXV BRI (Fig. 1A). These sequences relative to other VACV-like viruses essentially extend from the ITR boundary region to the region upstream of the HSPV016 viral growth factor homologue (CPN C11R), replacing the OPV-like sequence that is transposed from the right terminal region to the left terminal region in other VACV-like viruses. HSPV sequences in this region include 15 ORFs representing three intact OPV genes (HSPV008, HSPV010, and HSPV012) and six potentially truncated or fragmented genes (HSPV007, HSPV009, HSPV011a to -c, HSPV013, HSPV014a to -d, and HSPV015a and -b) (Table 1; Fig. 1). HSPV008 encodes an intact protein orthologous only to CPXV GRI-90 D7L and ECTV strain Moscow EVM004 (21, 79). These proteins contain amino-terminal BTB/POZ domains, evolutionarily conserved domains important for oligomerization and ordering of protein complexes and often present in amino-terminal regions of both cellular and poxviral kelch-like proteins, but in these smaller HSPV008 orthologues the BTB/POZ domain is not associated with kelch repeat domains (3, 75). HSPV009 encodes a truncated orthologue of CPXV GRI-90 D12L product, a protein similar to the CrmB carboxyl terminus and whose orthologue in ECTV was recently characterized as a secreted chemokine binding protein (7). HSPV010 encodes an intact orthologue of CD30 TNFR-like proteins present in CPXV and ECTV, proteins able to bind CD30 ligands and/or have immunomodulatory effects (63, 72). HSPV left-end sequences also contain genes for three ankyrin repeat proteins absent in VACV. While HSPV012 encodes an intact ankyrin repeat protein also intact in CPXV and MPXV, HSPV011a to -c and HSPV014a to -d encode fragments of intact ankyrin repeat proteins encoded only in ECTV and/or CPXV, with HSPV014b and -c encoded within the region containing 1.4 kbp of sequence found only in CPXV. Finally, HSPV015a and -b appeared to encode fragments of a paralogue of CPN C7L, a VACV host range protein which enables viral replication in human cells (67). While all OPVs appear to encode intact C7L orthologues (HSPV024), intact HSPV015 orthologues are encoded only in CPXV and CMLV, with fragmented ORFs annotated in MPXV and VARV (Table 1).

HSPV sequence in the right terminal region absent in other VACV-like viruses essentially bound the region homologous to the VACV WR SPI-1 (HSPV198) locus, a region transposed to the opposite terminus in several other VACVs (Fig. 1). Unique sequence upstream of HSPV198 includes HSPV197, an intact kelch-like protein also intact in CPXV and ECTV but fragmented or absent in MPXV, CMLV, and VARV. Unique sequences downstream of HSPV198 contain an intact orthologue of the VARV strain Bangladesh B22R gene (HSPV200). B22R homologues represent the largest poxviral genes, encoding proteins of approximately 2,000 amino acids and with no known function but predicted to contain carboxyl-terminal transmembrane domains and cysteine residues which conceivably mediate disulfide bond formation (54, 56, 76). B22R homologues are intact in all OPV species except VACV-like viruses, making the presence of HSPV200 notable (Fig. 1).

Despite containing additional sequence not present in other VACV-like viruses, HSPV did lack sequences homologous to several larger regions in other OPVs. These include from GRI-90 the D8L to D11L locus, a region encoding ankyrin repeat, kelch-like, and lectin-like proteins with homologous sequence only in ECTV (79) (Table 1), and most of the K1R to S1R/T1R locus, a region encoding ankyrin repeat, CrmD TNFR, and CrmE TNFR proteins and with homologous sequence present in MPXV, ECTV, and CMLV and, notably, in VACV Lister (Fig. 1C). HSPV also lacks any remnant of the second VARV B22R-like gene identified in certain strains of CPXV and of which remnants remain in VARV and CMLV lineages (HSPV185-HSPV186 locus [Fig. 1C]) (56).

Finally, HSPV contains fragmented genes intact in all or nearly all other VACV-like viruses. Within the central conserved region, HSPV074a to -d represented fragments of the CPN I4L ribonucleotide reductase large subunit gene, while HSPV044 encoded an intact small subunit (Table 2). Ribonucleotide reductase is a heterodimeric protein involved in redox reactions that are key to synthesis of deoxyribonucleotides, an activity for which various poxviruses encode different enzyme complements, potentially adapted to replication in specific host cell types lacking adequate nucleotide pools (59). Experimental disruption of the VACV ribonucleotide reductase large subunit has been shown previously to have no effect on virus replication in vitro and a mild effect on virulence in mice (23). Although I4L homologues are not encoded in all other poxviral genera, to our knowledge this is the first example of its natural disruption in an OPV genome. Similarly, HSPV183 is unique among VACV-like homologues (CPN B6R) as the only form of the gene to be fragmented, although a fragmented form is also found in VARV (Fig. 1B) and an isolate of MPXV (accession no. AAY97373). Notably, HSPV contained fragmented genes intact in all VACVs except MVA, a virus that has accumulated numerous mutations and extensive nucleotide deletions through extensive passage in vitro and concomitant attenuation and restriction of host range (9). These include HSPV026, orthologue of CPN C5L BTB domain protein, and the HSPV033 ankyrin repeat protein. In addition, HSPV178, similar to MVA, demonstrates a smaller fragmented form of the guanylate kinase gene than do other VACV-like viruses.

Perspective on relationship of HSPV to VACV. Genomic sequence analysis of HSPV MNR-76 indicates that it is a novel VACV-like OPV that contains unique features not present in known VACVs. Although MNR-76 is unique in the comple-

ment of OPV genes remaining intact in HSPV, the pattern of terminal gene loss/fragmentation is commensurate with genotypes observed in other VACV-like viruses. Notably, the majority of left terminal HSPV sequence absent in VACV appears to contain gene fragments, with HSPV conceivably in the process of losing this sequence similarly to other VACV-like viruses.

The close phylogenetic and genotypic relationship between HSPV and other VACV-like viruses and the presence of additional CPXV-like sequences in HSPV are notable given previous speculations involving horsepox and the origins of VACV (14). While the origins of current VACV-like strains have been heavily debated and remain obscure, current knowledge affirms that VACV-like viruses constitute an OPV lineage independent of known CPXV and VARV species from which VACV has been speculated to be derived (14, 32, 33, 38) (Fig. 2). It is likely that a once naturally circulating but now rare VACV-like virus(s) from which current strains are derived was introduced as a vaccine virus, and the agent of horsepox has been surmised as a likely candidate (14). Indeed, apparently Edward Jenner believed that his vaccine originated from the "grease" infection found in the heels of horses, and the use of horse-derived material for use as vaccines is documented (14, 33). In addition, phenotypic similarity of certain vaccines transmitted between cows, humans, and horses has been noted, and experimental infection of horses with VACV can produce clinical signs of horsepox (14, 44, 86). The data presented here indicate that the HSPV MNR-76 genome contains features consistent with such a hypothesis, a phylogenetically VACV-like virus isolated from a horse and containing additional OPV-like terminal sequences, sequences likely ancestral and absent in other VACV-like viruses yet in certain regions appearing to be undergoing gene fragmentation and loss commensurate with transition toward a VACV-like genotype.

Despite speculation as to what role horsepox played in the development of smallpox vaccines, it is clear that HSPV MNR-76 does not represent a direct ancestral genotype to all known VACVs, given the disruption of many HSPV genes intact in certain VACV isolates (Table 1). It is unclear what constitutes the genotypic diversity of all the viruses historically used for smallpox vaccine, especially considering the potential for disparate source material and passage histories of VACV-like vaccine viruses (14, 33). Indeed, phenotypic and genotypic diversity is observed between and within strains of VACV (14, 33, 58) (Fig. 1). This diversity does include sequence unique to a given strain, such as the presence of CPXV GRI K3R and S1R/T1R-like genes in the historically important Lister vaccine strain (Fig. 1C), making the presence of HSPV MNR-76-like sequences in uncharacterized vaccine strains a possibility. Isolated in 1976, HSPV was causing disease in horses while smallpox vaccines were still being distributed during the World Health Organization global smallpox eradication program (32). Conceivably, local or a currently uncharacterized vaccine could have been introduced into the horse population, as contact with vaccinated persons is known to have been a source of OPV disease in animals (33). Vaccine escape has been hypothesized to account for other VACV-like viruses occasionally isolated from domestic and sentinel animals, including RPXV, buffalopox in India, and viruses associated with zoonosis in South America; however, unique biological properties and/or inability to associate the isolate with vaccine virus has also led to suggestions that they are natural VACV isolates or VACV subspecies (19, 24, 25, 27, 33, 46, 90). Similarly, HSPV MNR-76 may represent a novel, naturally circulating virus and perhaps one for which the horse was an incidental host, just as other domestic and captive animals are not thought to be the reservoir for CPXV infection despite being susceptible to infection (13, 33). Unfortunately, little is known of the prevalence of disease associated with HSPV MNR-76 in Mongolia, either in horse or in human populations. Conceivably, MNR-76 may represent a naturally circulating member of the VACV lineage, as were viruses circulating among domestic animals in the era in which current VACV-like viruses were collected as vaccine. Whatever the historical relationship between HSPV MNR-76 and characterized VACV-like viruses may be, genomic sequence analysis of other VACV-like virus isolates may add perspective to the novel nature of HSPV relative to other viruses within the VACV lineage.

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ADDENDUM IN PROOF

Since completion of the analyses presented here, the genome sequences of several VACV clones derived from the Dryvax vaccine have become available. Preliminary analysis indicates that while most of the HSPV sequence reported here as absent in VACV was also absent in these clones, one (GenBank accession no. AY313848) contained nucleotide sequence and ORF fragments at the HSPV 197 locus, stressing the need for additional genomic sequence and analyses in examining the nature of VACV-like virus variability.

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