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The genomic sequence of ectromelia virus, the causative agent of mousepox

Nanhai Chen,[a,1] Maria I. Danila,[b,1] Zehua Feng,[a] R. Mark L. Buller,[a,*] Chunlin Wang,[c] Xiaosi Han,[c] Elliot J. Lefkowitz,[c] and Chris Upton[b]

[a] *Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center, 1402 South Grand Boulevard, St. Louis, MO 63104, USA*

[b] *Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 2Y2, Canada*

[c] *Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

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Abstract

Ectromelia virus is the causative agent of mousepox, an acute exanthematous disease of mouse colonies in Europe, Japan, China, and the U.S. The Moscow, Hampstead, and NIH79 strains are the most thoroughly studied with the Moscow strain being the most infectious and virulent for the mouse. In the late 1940s mousepox was proposed as a model for the study of the pathogenesis of smallpox and generalized vaccinia in humans. Studies in the last five decades from a succession of investigators have resulted in a detailed description of the virologic and pathologic disease course in genetically susceptible and resistant inbred and out-bred mice. We report the DNA sequence of the left-hand end, the predicted right-hand terminal repeat, and central regions of the genome of the Moscow strain of ectromelia virus (approximately 177,500 bp), which together with the previously sequenced right-hand end, yields a genome of 209,771 bp. We identified 175 potential genes specifying proteins of between 53 and 1924 amino acids, and 29 regions containing sequences related to genes predicted in other poxviruses, but unlikely to encode for functional proteins in ectromelia virus. The translated protein sequences were compared with the protein database for structure/function relationships, and these analyses were used to investigate poxvirus evolution and to attempt to explain at the cellular and molecular level the well-characterized features of the ectromelia virus natural life cycle.

Keywords: Poxvirus; Ectromelia virus; Virulence; Mousepox; Genomic sequence

Introduction

The poxvirus family is divided into two subfamilies: *Entomopoxvirinae* (poxviruses of insects) and *Chordopoxvirinae* (poxviruses of the vertebrates). Poxviruses replicate in the cytoplasm of cells and assemble large virions that are visible in the light microscope. The structurally complex virions contain a double-stranded DNA genome of between ~140 and 280 kb, and an early gene transcription system (Moss, 2001). Poxviruses are classified into genera by comparing cross-protection in animal studies, cross-neutralization of infectivity in tissue culture, and genomic DNA sequence information.

Genomic sequences have been determined for representative species from seven of the eight vertebrate poxvirus genera: *avipoxvirus* (fowlpox virus) (Afonso et al., 2000); *capripoxvirus* (lumpy skin disease virus, sheeppox virus, and goatpox virus) (Tulman et al., 2001, 2002); *leporipoxvirus* [shope fibroma virus (Willer et al., 1999) and myxoma virus (Cameron et al., 1999)]; *molluscipoxvirus* (molluscum contagiosum virus) (Senkevich et al., 1997); *orthopoxvirus* [monkeypox virus (Shchelkunov et al., 2002), camelpox virus (Afonso et al., 2001; Gubser and Smith, 2002), vaccinia virus (Goebel et al., 1990), and variola virus (Massung et al., 1994; Shchelkunov et al., 1995, 2000)]; *suipoxvirus* (swinepox virus) (Afonso et al., 2002); and *yatapoxvirus*

* Corresponding author. Fax: +1-314-773-3403.
E-mail address: Bullerrm@slu.edu (R.M.L. Buller).
[1] These authors contributed equally to this work.

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(Yaba-like disease virus) (Lee et al., 2001). The availability of these genomic sequences has permitted the comparison of distantly related orthologs to determine conserved amino acids (aa), which in turn can facilitate bioinformatics predictions as to ORF function.

Ectromelia virus (ECTV) is the causative agent of mousepox, an acute exanthematous disease of mouse colonies in Europe, Japan, China, and the U.S. (Buller and Palumbo, 1991; Fenner, 1982). The disease is on the decline in vivariums due to improvements in animal husbandry and health surveillance. The natural reservoir for ECTV is unknown but one report provides evidence that wild mice may be involved. Laboratory studies have shown ECTV to have a very narrow host range, infecting only certain mouse species (Buller et al., 1986; Fenner, 1982). A number of different strains of ECTV have been isolated which have been shown to differ in their virulence for the mouse. The Moscow, Hampstead, and NIH79 strains are the most thoroughly studied with the Moscow strain being the most infectious and virulent for the mouse. In the late 1940s mousepox was proposed as a model for the study of the pathogenesis of smallpox and generalized vaccinia in humans (Fenner, 1948). Studies in the last five decades from a succession of investigators have resulted in a detailed description of the virologic and pathologic disease course in genetically susceptible (A, BALB/c, DBA/2, and C3H/He) and resistant (C57BL/6 and AKR) inbred and outbred mice; identification and characterization of the important cell-mediated and innate responses for recovery from infection; and the discovery of *rmp-1*, *rmp-2*, *rmp-3*, and *rmp-4* loci, which govern resistance to severe mousepox (Brownstein and Gras, 1995; Brownstein, 1998; Delano and Brownstein, 1995). Since varying mouse genotype, virus strain, and dose of virus can manipulate disease patterns for a given route of infection, ECTV infections of mice are one of the best models for studying viral pathogenesis, and testing efficacy of orthopoxvirus antivirals and vaccines.

In this study we report the DNA sequence of the left-hand end, the predicted right-hand terminal repeat, and central regions of the genome of the Moscow strain of ECTV (approximately 177.5 kb), which together with the previously sequenced right-hand end provide the entire protein coding region of the genome (Chen et al., 2000). The translated protein sequences were compared with the protein database for structure/function relationships, and these analyses have been used to investigate poxvirus evolution and to explain at the cellular and molecular level the well-characterized features of the ECTV natural life cycle.

Results and discussion

Overview of the structure of the genome of ectromelia virus strain Moscow

We report 175,950 base pairs (bp) of contiguous sequence, which was assembled from 1832 independent sequencing reactions based on 16 overlapping PCR fragments generated from genomic DNA of ECTV strain Moscow (ECTV-MOS), and the predicted 1503-bp right-hand terminal repeat. Each position was sequenced on both strands at least once with an average coverage of 3.7-fold. This sequence, in addition to 32,318 bases sequenced previously, constitutes a genome of 209,771 bp (Chen et al., 2000). The presented sequence does not contain the hairpin termini of the ECTV-MOS genome, but based on DNA homology with closely related orthopoxvirus vaccinia [VACV, strain Copenhagen (COP)], the ECTV-MOS sequence presented here starts ~136 bases from the terminus. This value is consistent with the estimated position of the 5′ primer sequence containing a part of the concatamer resolution motif, ATTTAGTGTCTAGAAAAAAA, which was used to amplify the left terminal fragment, and restriction endonuclease analysis of the terminal region of the genome (Baroudy et al., 1982; Esposito and Knight, 1985; Goebel et al., 1990; Merchlinsky, 1990; Stuart et al., 1991). As is typical of the orthopoxviruses, the genome of ECTV-MOS is A+T-rich (67%), although there is considerable variation between individual ORFs. Previous DNA restriction enzyme mapping analyses have shown genomes of orthopoxviruses to have a central, highly conserved region. The region between ORF 029 (VACV-COP F6L) and ORF 127 (VACV-COP A24R), which covers 99,798 nucleotides, shows strong conservation of gene order, and approximately 97, 96, and 97% aa identity when compared to a similar region of VACV-COP, variola virus strain Bangladesh (VARV-BSH), and cowpox virus strain Brighton Red (CPXV-BR) counterparts.

The inverted terminal repeat (ITR) reported here contains 9413 bp. The right ITR junction falls within ORF 169 and thus a C-terminal fragment of this ORF, designated as the noncoding Region D, is present in the left ITR (Fig. 1). There are several specific structures contained in the orthopoxvirus ITR: terminal hairpin, the concatamer resolution motif, two conserved nonrepeating sequences, NR I and NR II, and direct repeats on each side of the NR II region. An alignment of the ITRs from VACV-COP, VARV-BSH, cowpox virus strain Grishak (CPXV-GRI), and ECTV-MOS shows that NR I and NR II regions are highly conserved within these genomes (data not shown). The DR I of ECTV-MOS, named for convention, is not in fact repeated but contains one copy of a 68-bp element, which is similar to the 70-bp element from the ITR of VACV-COP, and one copy of a 25-bp element which is a portion of the 54-bp element in VACV-COP. The second set of direct repeats is represented by an 85-bp monomer, which is repeated 10.4 times. The 85-bp element can be aligned with the 54-bp element from VACV-COP, by introducing a 31 nucleotide insertion between positions 27 and 28 of the VACV-COP element (data not shown). This 85-bp monomer is polymorphic at position 30 of the unit, where either an A or a G can be present. Three genes (ORFs 001, 002, and 003) are encoded within the left-hand ITR of the genome and there-

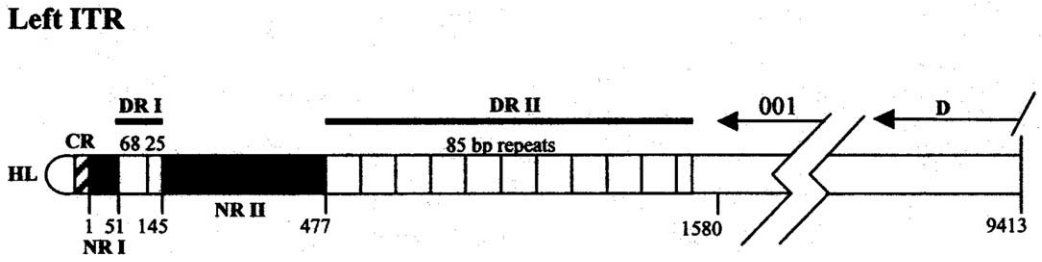

Fig. 1. Schematic representation of the left ITR of ECTV-MOS. The presumed hairpin loop (HL), concatemer resolution sequence (CR; hatched box), and the nonrepeating regions NR I and NR II, which are highly conserved among VACV, VARV, and CPXV are indicated. Nucleotide numbers for this sequence are shown at the bottom of the diagram. On each side of NR II are the direct repeat regions, DR I and DR II. The length of the repeat elements is indicated under the thick bars. The relative positions of the first leftmost ORF (001) and the ORF-disrupted region D are shown. The left ITR ends at position 9413.

fore are duplicated in the right-hand ITR (ORFs 170, 171, and 172; Fig. 2).

The ECTV-MOS genome has two unique features when compared to other orthopoxviruses. A small intergenic region in the ECTV-MOS genome, between nucleotides 16,879 (ORF 010) and 17,146 (ORF 010.5), contains several different short AT-rich direct repeats with copy numbers of 2–14. Very similar, but not identical repeats are also present in the Naval strain of ECTV (ECTV-NAV), indicating that the repeats probably arose early after speciation

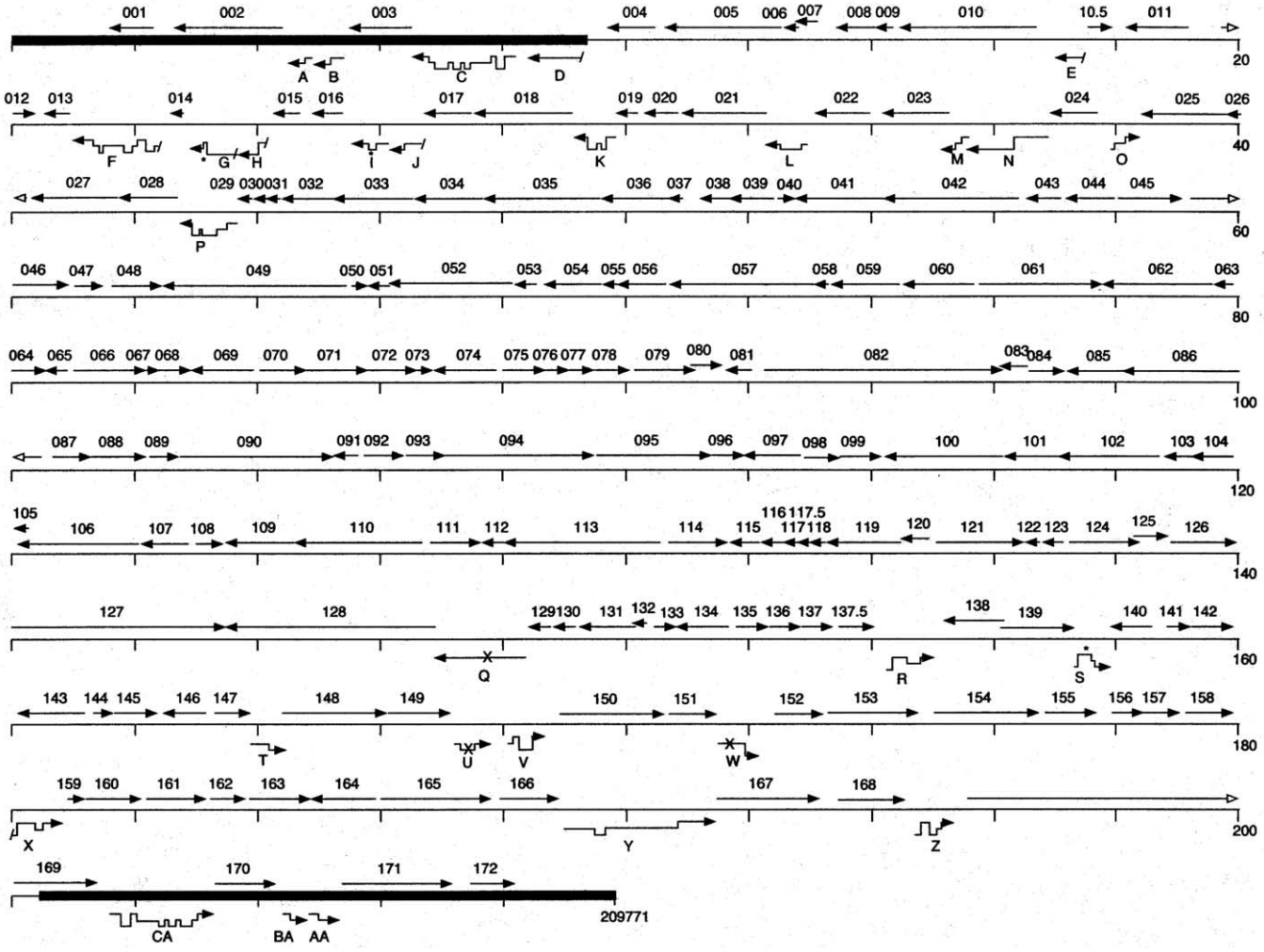

Fig. 2. ORF map of the ECTV-MOS genome. ORF map of ECTV strain Moscow genome. Scale in kb. 001 Named ORF; direction shown by arrow; Fragmented ORFs; readingframe shifts required to complete genes are shown; *indicates not all frameshifts shown; X indicates STOP codon; Significant deletion at 5′ end of ORF; ORF wraps around; Inverted terminal repeat.

Table 1
Regions (from Fig. 2) of ECTV-MOS genome containing fragments of genes present in other orthopoxviruses

Region	Start	Stop	Orthologous ORF	aa	Putative function/motifs
A	4823	4621	CPXV-BR 008	672	Ankyrin repeats
B	5428	4975	CPXV-GRI K3R	167	CrmE; TNFR homolog
C	8157	6475	CPXV-BR 220	579	Ankyrin repeats
D	9413	8435	ECTV-MOS 169	1924	Membrane protein; disrupted by ITR junction
E	17395	17168	CPXV-BR 017	435	Ankyrin repeats
F	22467	20974	CPXV-BR 025	668	CPXV CHO host range
G	23735	22958	VACV-COP C9L	634	Ankyrin repeats
H	24183	23743	VACV-COP C8L	184	Unknown
I	26139	25535	VACV-COP C5L	204	Unknown
J	26745	26207	VARV-BSH D14L	316	Unknown
K	29859	29224	CPXV-BR 036	231	Unknown
L	32952	32290	VACV-COP M2L	220	Unknown
M	35581	35357	VACV-COP K3L	88	eIF-2α homologue
N	36910	35632	VACV-COP K4L	424	Phospholipase D-like
O	37903	38348	VACV-COP K7R	149	Unknown
P	43752	42709	VARV-BSH C9L	348	Unknown
Q	148391	146880	VARV-BSH A30L	498	P4c, inclusion factor
R	154219	154984	VACV-COP A37R	263	Unknown
S	157331	157879	VACV-COP A40R	168	Lectin (NK cell receptor)
T	163913	164403	VACV-COP A49R	162	Unknown
U	167228	167773	VACV-COP A52R	190	inhibits NK-κB activation
V	168098	168657	CPXV-BR 191	186	CrmC; TNFR homologue
W	171533	172125	CPXV-BR 195	197	Guanylate kinase
X	179961	180812	CPXV-BR 204	501	Unknown
Y	189026	191413	CPXV-BR 213	800	Ankyrin repeats
Z	194724	195303	CPXV-BR 218	193	Similarity to VACV-COP C13L and C14L
CA	201615	203297	CPXV-BR 220	579	Ankyrin repeats
BA	204344	204797	CPXV-GRI K3R	167	CrmE; TNFR homologue
AA	204949	205151	CPXV-BR 008	672	Ankyrin repeats

and that they represent a dynamic region of the genome (http://www.sanger.ac.uk/Projects/Ectromelia-virus/). Deletions between short direct repeats appear to be a common feature in poxvirus genomes (Upton et al., 1994a), but nothing is known about the mechanism by which the deletions occur or how some repeated regions appear to be maintained. After position 157,776 between ORFs 139 and 140, sequence analysis of PCR amplicons identified a C tract that was heterogeneous in nature. Individual DNA molecules from these PCR-amplicons were cloned and DNA sequence analysis revealed five clones with 18 Cs, two clones with 19 Cs, and one clone each with 16 Cs, 17 Cs, and 20 Cs. To confirm the heterogeneity of this region in the genomic DNA, two independent ECTV-MOS genomic DNA clones were sequenced. Clones E23 and B27 were found to have 19 and 21Cs, respectively. In the submitted GenBank file, the sequence was reported with 21 Cs starting at nucleotide 157,777. The ECTV-NAV and ECTV-WIE (accession No. AF 043238) strains have 11 and 8 Cs in this position, respectively, confirming another highly polymorphic site in the ECTV genome. VACV (strains COP, TIA, ANK, and WR) and VARV (strains IND, GAR, and BSH) have three Cs in this position, whereas CPXV-BR has four Cs one of which may represent a T $\rightarrow$ C mutation. The C tract is present in the NK cell receptor homologue (VACV-A40R) in the genomes of VACV strains, but within the intergenic region in the genomes of strains of VARV because the orthologs are truncated.

ORFs predicted to encode proteins of at least 60 aa have been assigned gene numbers starting from the left end of the genome as aligned to VACV-COP. To increase the accuracy of the annotation process, we have examined predicted ORFs for experimental evidence of expression in other orthopoxviruses, the presence of proximal transcription initiation and termination signals, and aa content and pI of the predicted proteins (Upton, 2000). We have not annotated small ORFs that significantly overlap with ORFs that are well conserved in other poxviruses. We have separately annotated regions of the ECTV-MOS genome that contain gene fragments (alphabetically), rather than ascribing them gene numbers (Fig. 2 and Table 1). An assignment as a gene fragment was based on comparisons to poxvirus ORFs in the database. To show the likelihood of a region producing a fragment of the normal encoded protein, the nature of the fragmentation is illustrated in Fig. 2. Using this approach we identified 175 potential genes specifying proteins of between 53 and 1924 aa, and 29 regions containing sequences related to genes predicted in other poxviruses but unlikely to encode for functional proteins in ECTV-MOS. Although our primary cutoff for predicted ORFs was 60 aa, we have included two small ORFs, 014 and 117.5, due to the existence of orthologs in a number of other poxvirus

genomes. As in many other poxviruses, there is a strong preference for ORFs within about 50 kb of the genomic termini (~40 ORFs) to be transcribed toward the closest end of the genome. Fig. 2 indicates that the ORFs are closely packed within the ECTV-MOS genome.

Essential genes for DNA replication, transcription, and virion assembly

ECTV-MOS contains a complement of genes that are orthologs of most of the orthopoxvirus genes involved in virus replication, nucleotide metabolism, virion structure, and assembly (Table 2). Examination of this group of essential genes found ORF 137.5 to contain three separate deletions that resulted in three translational frameshifts of the predicted protein such that its length was 160 aa instead of the 221 predicted for the VACV-COP A36R. The first deletion of 5 bp after nucleotide 390 of the gene shifted the reading frame, and after 12 more bp a second deletion of 4 bp restored the original reading frame that continued for 61 bp before a third 1 bp deletion. This final deletion resulted in six more out-of-frame aa and a STOP codon. An identical pattern of deletions in the A36R homologue was also observed in ECTV-MP1 (Accession No. AJ315003). The A36R homologue is an integral intracellular enveloped virus (IEV) membrane protein important for the microtubule-mediated intracellular movement of IEV, and actin tail formation, which is required for release of extracellular enveloped virions (EEV). Also an A36R homologue was important for maximum virulence of VACV-WR in a murine intranasal model (Parkinson and Smith, 1994). Previous studies showed that residues 1–100 of A36R were sufficient to mediate microtubule-based motility of VACV IEVs to the cell periphery, and residues 1–117 were sufficient for actin tail formation, but at reduced levels (Frischknecht et al., 1999; Rietdorf et al., 2001). It is likely that the ECTV-MOS homologue of A36R is at least partially functional as ECTV-MOS produces comet-shaped plaques under liquid overlay (M. Buller, unpublished data), and ECTV-MOS causes the disease mousepox in inbred and outbred strains of mice following infection by multiple routes.

A-type inclusion body protein

A-type inclusions (ATIs) are acidophilic cytoplasmic masses also known as Marchal and Downie bodies in ECTV and CPXV infections, respectively. ATIs result from the condensation of a major protein species (160 kDa in CPXV-BR) expressed in large amounts at late times during some poxvirus infections (Funahashi et al., 1988). There are three types of ATIs, distinguished by the amount and location of associated virions. Some ATIs lack virions altogether (V^- character), whereas others have particles throughout (V^+) or only along the periphery (V^i) (Shida et al., 1977). Occlusion of virions into the ATI is dependent on the presence of the p4c gene product (VARV-BSH A30L) on the surface of IMV particles, which may play a role in the retrograde movement of IMV into ATIs. Thus p4c may affect the overall balance of EEV, IMV, and occluded IMV particles produced in an infected cell, and thus play a role in virus dissemination in vivo (McKelvey et al., 2002). The p4c homologue in the ECTV-MOS (Table 1, Region Q) and NAV strains is fragmented, and therefore, these ECTV isolates would not be expected to occlude IMV. ATIs may provide a means of survival in the environment as is the case with insect poxviruses' dependence on an inclusion structure called a spherule (Bergoin and Dales, 1971). Alternatively, macrophages may be more efficiently infected when virus is released in the low-pH endocytic vesicles or lysosomal compartments following phagocytosis of ATIs containing virus (Ulaeto et al., 1996). Although both possibilities are plausible, it is puzzling why different strains of CPXV isolated directly from nature vary in the ability to occlude virions in ATIs (Baxby, 1975). ORF 128 encodes an A-type inclusion protein of 1113 aa. Currently, only ECTV and CPXV are known to code for a complete ATI protein (Funahashi et al., 1988); other poxviruses such as VACV-COP and VARV-BSH contain only fragments of the ATI. ECTV-MOS and ECTV-NAV ATI proteins are 100 and 97.5% identical to the ATI of ECTV strain MP-1, which is 64 residues shorter (Accession No. X69325) (Osterrieder et al., 1994). ECTV-MOS and CPXV-BR ATI genes are approximately 85% identical at the nucleotide level, but the proteins are less similar than expected because a series of small deletions result in reading frameshifts in some regions of the proteins. There are, however, regions where significant portions of the proteins are more than 95% homologous.

Epidermal growth factor homologues

Vaccinia virus growth factor (VGF), an epidermal growth factor (EGF) homologue, has been found in the genomes of all sequenced leporipoxviruses and orthopoxviruses. Studies using VACV have suggested that VGF stimulates quiescent cells proximal to the focus of infection to proliferate, and perhaps serves as better hosts for subsequent rounds of infection. VGF homologues of myxoma virus (MYXV-LAU) and VACV-WR have been found to be important for optimal virulence in rabbits, mice, and chick embryos (Buller et al., 1988a,b; Opgenorth et al., 1992). In comparison to the fully processed 77 aa VGF (VACV-COP C11R), the EGF-like protein encoded by ORF 10.5 and the identical homologue in ECTV-NAV are predicted to have a processed length of 65 aa. This truncated protein, however, may be functional because the termination codon is 4 aa after the last of six highly conserved cysteines that are believed to be important for structure of the protein and results in a polypeptide that is only 5 aa shorter than the functional MYXV EGF-like proteins (Chang et al., 1990; Opgenorth et al., 1993). In contrast to the ECTV-MOS and NAV strains, a gene encoding an EGF-like protein from the

Table 2
Predicted ORFs in ECTV-MOS

Ectromelia virus strain Moscow					Similarity to orthologs			
Name	aa	Start	Stop	Predicted features/function	VACV-COP	aa	% Identity	VARV-BSH
001	247	2323	1580	Sec/CC-Chemokine bp	C23L	244	81	Yes
002	587	4415	2652	Ankyrin repeats/unknown	BSH G1R	585	91	Yes
003	320	6468	5506	Sec/TNF-bp (CrmD)	BR 221	320	96	No
004	273	10,494	9673	BTB domain/unknown	GRI D7L	273	88	No
005	650	12,565	10,613	Ankyrin repeats/unknown	BR 011	658	93	No
006	75	12,824	12,597	C-type lectin/unknown	GRI D9L	75	77	No
007	103	13,077	12,766	Sec, C-type lectin/unknown	GRI C2L	96	91	No
008	202	14,019	13,411	Sec/TNFR C-terminal domain	BR 014	202	92	No
009	111	14,351	14,016	Sec/vCD30	BR 015	110	90	No
010	763	16,733	14,442	Ankyrin repeats/unknown	BR 016	764	91	No
010.5	83	17,589	17,840	Shortened EGF-like factor	C11L	142	78	Yes
011	331	19,149	18,154	Unknown	C10L	331	98	Yes
012	241	19,658	20,383	Zinc finger/apoptosis	BSH D6R	242	95	Yes
013	138	20,915	20,499	IL-18bp	BSH D7L	126	94	Yes
014	59	22,782	22,603	Unknown	BR 026	63	87	No
015	150	24,700	24,248	Ankyrin repeats/host range	C7L	150	95	Yes
016	155	25,391	24,924	Unknown	C6L	151	90	Yes
017	262	27,564	26,776	Sec/complement-bp control/CD46	C3L	263	93	Yes
018	512	29,158	27,620	Kelch repeats/unknown	C2L	512	97	Yes
019	117	30,199	29,846	Cytoplasmic protein/virulence	N1L	117	89	Yes
020	177	30,861	30,328	α-Amanitin sensitivity	N2L	175	91	Yes
021	472	32,312	30,894	Ankyrin repeats/unknown	M1L	472	96	Yes
022	285	33,944	33,087	Ankyrin repeats/host range	K1L	284	97	Yes
023	373	35,291	34,170	Serine protease inhibitor/SPI-3	K2L	369	91	Yes
024	277	37,765	36,932	Lysophospholipase/unknown	BR 045	276	96	No
025	456	39,789	38,419	Unknown	F1L	226	85	Yes
026	147	40,232	39,789	Deoxyuridine triphosphatase	F2L	147	97	Yes
027	482	41,708	40,260	Kelch repeats/unknown	F3L	480	95	Yes
028	319	42,678	41,719	Ribonucleotide reductase-S	F4L	319	99	Yes
029	74	43,927	43,703	Unknown	F6L	74	93	Yes
030	82	44,190	43,942	Unknown	F7L	92	80	Yes
031	65	44,528	44,331	Proline rich/not essential	F8L	65	97	Yes
032	212	45,224	44,586	Membrane/unknown	F9L	212	99	Yes
033	439	46,530	45,211	Serine, threonine kinase	F10L	439	99	Yes
034	354	47,617	46,553	Unknown	F11L	354	97	Yes
035	634	49,580	47,676	IEV actin tail formation	F12L	635	96	Yes
036	372	50,731	49,613	IEV, CEV, EEV membrane	F13L	372	99	Yes
037	71	50,964	50,749	Unknown	F14L	73	78	Yes
038	158	51,705	51,229	Unknown	F15L	158	99	Yes
039	231	52,407	51,712	Membrane/unknown	F16L	231	95	Yes
040	101	52,469	52,774	Virion DNA-bound phosphoprotein	F17R	101	98	Yes
041	479	54,210	52,771	Poly(A) polymerase-L	E1L	479	99	Yes
042	737	56,420	54,207	Unknown	E2L	737	98	Yes
043	190	57,098	56,526	dsRNA-binding/PKR+OAS inhibitor	E3L	190	93	Yes
044	259	57,934	57,155	RNA polymerase, 30-kDa, VITF-1	E4L	259	100	Yes
045	331	58,015	59,010	Virosome component	E5R	331	92	Yes
046	567	59,211	60,914	Unknown	E6R	567	98	Yes
047	165	60,995	61,492	Myristylated/unknown	E7R	166	93	No
048	273	61,620	62,441	Membrane/unknown	E8R	273	98	Yes
049	1006	65,468	62,448	DNA polymerase	E9L	1006	98	Yes
050	95	65,500	65,787	IMV membrane/-S-S-bond pathway	E10R	95	98	Yes
051	129	66,171	65,782	IMV core	E11L	129	95	Yes
052	666	68,158	66,158	Membrane/unknown	O1L	666	97	Yes
053	108	68,528	68,202	Glutaredoxin/unknown	O2L	108	99	Yes
054	312	69,610	68,672	IMV core, morphogenesis	I1L	312	98	Yes
055	73	69,838	69,617	Membrane/unknown	I2L	73	100	Yes
056	269	70,648	69,839	ssDNA-binding/phosphoprotein	I3L	269	98	Yes
057	771	73,046	70,731	Ribonucleotide reductase-L	I4L	771	97	Yes
058	79	73,312	73,073	IMV membrane	I5L	79	97	Yes
059	382	74,479	73,331	Unknown	I6L	382	99	Yes
060	423	75,743	74,472	IMV core, cysteine proteinase	I7L	423	98	Yes

Table 2 (*continued*)

Ectromelia virus strain Moscow					Similarity to orthologs			
Name	aa	Start	Stop	Predicted features/function	VACV-COP	aa	% Identity	VARV-BSH
061	676	75,749	77,779	RNA helicase/NPH-II	I8R	676	97	Yes
062	591	79,557	77,782	Putative metalloproteinase	G1L	591	99	Yes
063	111	79,889	79,554	Sec/Unknown	G3L	111	97	Yes
064	220	79,883	80,545	VLTF	G2R	220	98	Yes
065	124	80,889	80,515	IMV membrane, -S-S-bond pathway	G4L	124	99	Yes
066	434	80,892	82,196	Unknown	G5R	434	97	Yes
067	63	82,204	82,395	RNA polymerase, 7-kDa subunit	G5, 5R	63	100	Yes
068	165	82,397	82,894	Unknown	G6R	165	96	Yes
069	371	83,974	82,859	IMV core, matrix?	G7L	371	99	Yes
070	260	84,005	84,787	Late transcript factor, VLTF-1	G8R	260	100	Yes
071	340	84,807	85,829	Myristylated/unknown	G9R	340	97	Yes
072	250	85,830	86,582	IMV membrane, neut ab	L1R	250	99	Yes
073	87	86,614	86,877	Membrane/unknown	L2R	87	97	Yes
074	346	87,907	86,867	Unknown	L3L	350	94	Yes
075	251	87,932	88,687	IMV core, ssDNA binding	L4R	251	99	Yes
076	128	88,697	89,083	Membrane/unknown	L5R	128	100	Yes
077	153	89,040	89,501	IMV membrane, morphogenesis	J1R	153	99	Yes
078	177	89,517	90,050	Thymidine kinase	J2R	177	96	Yes
079	333	90,115	91,116	Poly(A) polymerase-S	J3R	333	99	Yes
080	185	91,031	91,588	RNA polymerase, 22-kDa subunit	J4R	185	99	Yes
081	133	92,054	91,653	Membrane/essential	J5L	133	98	Yes
082	1286	92,160	96,020	RNA polymerase, 147-kDa subunit	J6R	1286	99	Yes
083	171	96,532	96,017	Tyrosine/Serine phosphatase	H1L	171	99	Yes
084	189	96,546	97,115	Membrane/unknown	H2R	189	98	Yes
085	324	98,092	97,118	IMV membrane, surface, attach	H3L	324	96	Yes
086	794	1,00,477	98,093	RNA pol-associated prot, RAP 94	H4L	795	99	Yes
087	212	1,00,663	1,01,301	Late transcript factor, VLTF-4	H5R	203	93	Yes
088	314	1,01,302	1,02,246	DNA topoisomerase type I	H6R	314	99	Yes
089	146	1,02,283	1,02,723	Membrane/unknown	H7R	146	96	Yes
090	843	1,02,765	1,05,296	MRNA capping enzyme-L	D1R	844	98	Yes
091	146	1,05,695	1,05,255	IMV core	D2L	146	99	Yes
092	237	1,05,688	1,06,401	IMV core	D3R	237	97	Yes
093	218	1,06,401	1,07,057	Uracil-DNA glycosylase	D4R	218	98	Yes
094	785	1,07,089	1,09,446	Nucleoside triphosphatase	D5R	785	99	Yes
095	637	1,09,487	1,11,400	Early transcription factor-S	D6R	637	99	Yes
096	161	1,11,427	1,11,912	RNA polymerase, 18-kDa subunit	D7R	161	99	Yes
097	304	1,12,789	1,11,875	IMV membrane, attach, neut ab	D8L	304	95	Yes
098	213	1,12,831	1,13,472	MutT-like/unknown	D9R	213	99	Yes
099	250	1,13,469	1,14,221	MutT-like/unknown	D10R	248	97	Yes
100	631	1,16,113	1,14,218	NPH-1	D11L	631	99	Yes
101	287	1,17,010	1,16,147	MRNA capping enzyme-S	D12L	287	100	Yes
102	551	1,18,696	1,17,041	Virion morphogenesis, rif resist	D13L	551	99	Yes
103	150	1,19,172	1,18,720	Late transcript. factor, VLTF-2	A1L	150	99	Yes
104	224	1,19,867	1,19,193	Late transcript. factor, VLTF-3	A2L	224	99	Yes
105	76	1,20,094	1,19,864	Thioredoxin/-S-S-bond pathway	A2.5L	76	94	Yes
106	644	1,22,043	1,20,109	IMV core, precursor of p4b	A3L	644	99	Yes
107	281	1,22,941	1,22,096	IMV matrix, morphogenesis	A4L	281	94	Yes
108	164	1,22,979	1,23,473	RNA polymerase, 22 and 21-kDa	A5R	164	99	Yes
109	372	1,24,588	1,23,470	Unknown	A6L	372	99	Yes
110	710	1,26,744	1,24,612	Early transcription factor-L	A7L	710	99	Yes
111	288	1,26,798	1,27,664	Intermed transcript, VITF-3-S	A8R	288	99	Yes
112	110	1,27,993	1,27,661	IMV membrane, morphogenesis	A9L	99	85	Yes
113	891	1,30,669	1,27,994	IMV core, precursor of p4a	A10L	891	97	Yes
114	318	1,30,684	1,31,640	Membrane/unknown	A11R	318	99	Yes
115	191	1,32,217	1,31,642	IMV core	A12L	192	96	Yes
116	66	1,32,441	1,32,241	IMV membrane	A13L	70	84	Yes
117	90	1,32,822	1,32,550	IMV membrane, morphogenesis	A14L	90	99	Yes
117.5	53	1,33,000	1,32,842	IMV membrane, virulence	A14.5L	53	100	Yes
118	94	1,33,274	1,32,990	Unknown	A15L	94	98	Yes
119	377	1,34,391	1,33,258	Myristylated/unknown	A16L	378	97	Yes
120	202	1,35,002	1,34,394	IMV membrane, morphogenesis	A17L	203	96	Yes
121	493	1,35,017	1,36,498	DNA helicase	A18R	493	98	Yes
122	77	1,36,712	1,36,479	Unknown	A19L	77	97	Yes

(*continued on next page*)

Table 2 (*continued*)

Ectromelia virus strain Moscow					Similarity to orthologs			
Name	aa	Start	Stop	Predicted features/function	VACV-COP	aa	% Identity	VARV-BSH
123	118	1,37,069	1,36,713	Sec/Unknown	A21L	117	97	Yes
124	426	1,37,068	1,38,348	DNA pol processivity factor	A20R	426	98	Yes
125	187	1,38,278	1,38,841	Holliday junction resolvase	A22R	176	99	Yes
126	382	1,38,861	1,40,009	Intermed transcript, VITF-3-L	A23R	382	98	Yes
127	1164	1,40,006	1,43,500	RNA polymerase, 132-kDa subunit	A24R	1164	99	Yes
128	1113	1,46,834	1,43,493	A-type inclusion body	BR 158	1284	58	No
129	110	1,48,775	1,48,443	IMV membrane, attach, neut ab	A27L	110	95	Yes
130	146	1,49,216	1,48,776	Sec/transmembrane/unknown	A28L	146	97	Yes
131	305	1,50,134	1,49,217	RNA polymerase, 35-kDa subunit	A29L	305	98	Yes
132	77	1,50,330	1,50,097	IMV matrix, morphogenesis	A30L	77	96	Yes
133	126	1,50,490	1,50,870	Unknown	A31R	124	90	Yes
134	269	1,51,649	1,50,840	NTP-binding/DNA packaging	A32L	300	99	Yes
135	185	1,51,767	1,52,324	CEV, EEV membrane surface	A33R	185	90	Yes
136	168	1,52,348	1,52,854	CEV, EEV membrane surface	A34R	168	95	Yes
137	176	1,52,897	1,53,427	Unknown	A35R	176	96	No
137.5	160	1,53,495	1,53,977	IEV membrane	A36R	221	91	Yes
138	277	1,56,092	1,55,259	Membrane, CD47/not essential	A38L	277	97	Yes
139	399	1,56,107	1,57,306	Sec/Semaphorin-like	A39R	403	93	Yes
140	223	1,58,609	1,57,938	Sec/immunomodulator	A41L	219	91	Yes
141	134	1,58,771	1,59,175	Profilin-like	A42R	133	91	Yes
142	211	1,59,215	1,59,850	Membrane/unknown	A43R	194	66	Yes
143	346	1,61,192	1,60,152	Hydroxysteroid dehydrogenase	A44L	346	94	Yes
144	125	1,61,240	1,61,617	Superoxide dismutase-like	A45R	125	96	Yes
145	240	1,61,607	1,62,329	Inhibits NF-κB activation	A46R	214	98	Yes
146	244	1,63,150	1,62,416	Unknown	A47L	244	96	Yes
147	227	1,63,180	1,63,863	Thymidylate kinase	A48R	204	98	Yes
148	554	1,64,436	1,66,100	DNA ligase	A50R	552	97	Yes
149	334	1,66,153	1,67,157	Unknown	A51R	334	94	Yes
150	563	1,68,929	1,70,620	Kelch repeats/unknown	A55R	564	94	Yes
151	281	1,70,670	1,71,515	CEV, EEV membrane, hemagglutinin	A56R	315	80	Yes
152	299	1,72,283	1,73,182	Serine, Threonine kinase	B1R	300	97	Yes
153	503	1,73,249	1,74,760	Schlafen-like/unknown	BR 197	505	91	No
154	564	1,74,993	1,76,687	Ankyrin repeats/unknown	B4R	558	94	Yes
155	317	1,76,774	1,77,727	CEV, EEV membrane, neut ab	B5R	317	94	Yes
156	177	1,77,879	1,78,412	Membrane/unknown	B6R	173	88	Yes
157	181	1,78,451	1,78,996	ER protein/virulence	B7R	182	96	No
158	266	1,79,051	1,79,851	Sec/IFN-γ bp	B8R	272	92	Yes
159	87	1,80,883	1,81,146	Unknown	B11R	88	93	No
160	286	1,81,212	1,82,072	Serine, Threonine kinase	B12R	283	94	No
161	344	1,82,164	1,83,198	Serine protease inhibitor/SPI-2	B14R	222	95	Yes
162	164	1,83,277	1,83,771	Membrane/unknown	B15R	149	97	Yes
163	328	1,83,858	1,84,844	Sec/membrane protein/IL-1β bp	B16R	290	83	No
164	340	1,85,921	1,84,899	Unknown	B17L	340	94	Yes
165	594	1,86,011	1,87,795	Ankyrin repeats/unknown	B18R	574	93	Yes
166	358	1,87,856	1,88,932	Sec/cell surface IFN-α bp	B19R	353	88	Yes
167	559	1,91,522	1,93,201	Kelch repeats/unknown	A55R	564	25	Yes
168	370	1,93,460	1,94,572	Serine protease inhibitor/SPI-1	C12L	353	94	Yes
169	1924	1,95,563	2,01,337	Membrane/unknown	BSH B22R	1897	91	Yes
170	320	2,03,304	2,04,266	Sec/TNF bp (crmD)	BR 221	320	96	Yes
171	587	2,05,357	2,07,120	Ankyrin repeats/unknown	BSH G1R	585	91	Yes
172	247	2,07,449	2,08,192	Sec/CC-chemokine bp	C23L	244	81	Yes

Note. Name, ORF name; aa, number of amino acids; Start, first nucleotide of start codon; Stop, last nucleotide of stop codon; Similarity to orthologs: VACV-COP, name of VACV-COP ortholog, if no vaccinia ortholog, ORFs were compared to VARV-BSH (BSH) or CPXV (strains BR and GRI); aa, number of amino acids in ortholog; % identity, amino acid comparison; VARV-BSH, presence of ortholog. bp, binding protein; sec, secreted; BTB, (Broad-Complex, Tramtrack and Bric a brac) also known as the POZ domain (Poxvirus and zinc finger); cys, cysteine; S, small; IMV, intracellular mature virion; IEV, intracellular enveloped virion; EEV, extracellular enveloped virion; L, large; PKR, dsRNA-dependent protein kinase; OAS, 2′-5′-oligoadenylate synthetase; VLTF, viral late transcription factor; pol, polymerase; neut ab, neutralizing antibody; attach, attachment; rif resist, rifampicin resistance; NPH I, nucleoside triphosphate phosphohydrolase I; VITF, viral intermediate transcription factor; IEV, intracellular enveloped virion; gp, glycoprotein. Predictions: secreted proteins by SignalP V1.1; membrane proteins by TMpred.

putative Brazilian ECTV ICB isolate (Accession No. AAF21105) was predicted to have a primary translation product of similar length to VGF, and had 95 and 77% aa identity to VGF from VACV-COP and ECTV-MOS, respectively. Thus the ICB isolate may in fact be a VACV.

Schlafen-like protein

ORF 153, and its identical NAV strain homologue, is predicted to encode a 503 aa protein. The carboxyl-terminal ~300 aa is 34–43% identical to members of a family of mouse schlafen proteins. The ECTV-MOS schlafen protein increases the number of schlafen-like proteins in poxvirus species to four, since similar proteins are encoded by CMLV-M-96 (ORF 179) and CMLV-CMS (ORF 176R), CPXV-BR (ORF 197), and MPXV-ZAI (ORF B4R). The viral schlafen-like proteins demonstrate conservation of size and aa composition, showing that they are closely related and most likely originated from a common ancestor. Mouse schlafen proteins are thought to be involved in the regulation of T cell growth and development (Schwarz et al., 1998). Therefore the poxviral homologues might interfere with the ability of the host to generate an efficient cytotoxic immune response against the viral infection, although there is no experimental data to support this hypothesis. The ORF 153 equivalent in VACV COP and TIA strains are the adjacent B2R and B3R ORFs, and in VARV-BSH a series of three small ORFs (B2L, B3L, and B4L) and "noncoding" sequence.

Ankyrin repeat protein family

All orthopoxviruses sequenced so far contain several ORFs with predicted ankyrin repeat motifs. Ankyrin repeats are thought to be important for protein–protein interaction events between integral membrane proteins and cytoskeletal proteins (Andrade et al., 2001). Moreover, poxvirus proteins containing ankyrin-like motifs are thought to influence virus host range and pathogenesis. The ECTV-MOS ankyrin repeat protein family contains eight distinct members: ORF 002/171, ORF 005, ORF 010, ORF015, ORF 021, ORF 022, ORF 154, and ORF 165. BLASTP searches demonstrate that all of these ORFs have orthologs in other orthopoxvirus genomes with greater than 90% identity. The ancestral ECTV ankyrin repeat protein family may have contained six more members. Region A/AA contain a homologue of CPXV-BR 008 that is disrupted by a frameshift and a large deletion at the 3′ end. Region C/CA shows a high degree of relatedness to CPXV-BR 220, but has nine frameshifts and several deletions of varying length. Region E aligns with the last 74 aa sequence of CPXV-BR ORF 017, but contains a predicted three-frame translation product. The protein sequence of Region F is related to CPXV-BR ORF 025 that encodes the CPXV-BR CHO host range gene. Region G shows strong homology with VACV-COP C9L, but has undergone a large deletion at the 5′ end, and three frameshifts. The last potential ankyrin family member is located in the right terminal part of the genome where a three-frame translation of ECTV-MOS Region Y aligns with the protein sequence of CPXV-BR ORF 213.

Kelch repeat protein family

ECTV-MOS contains four genes that are predicted to encode proteins containing kelch repeats (Interpro Accession No. IPR001798; http://www.ebi.ac.uk/interpro/): ORF 018, ORF 027, ORF 150, and ORF 167. ORF 167 was previously designated as EV p65 or EV C13R. The first three ORFs have homologues in VACV-COP, and all four are present in CPXV-BR that also has two additional kelch-like genes. The biological functions of these poxvirus proteins are unknown; other proteins containing kelch motifs are involved in a variety of activities including interaction with microfilaments of actin (Totmenin et al., 2002). The kelch motifs are usually found as β-sheets that fold into higher order structures such as β-propellers or superbarrels (Pfam01344; in Protein Families Database of Alignments and Hidden Markov Models; http://www.sanger.ac.uk/Software/Pfam/).

Lectin motif containing proteins

ORFs 006 and 007 contains motifs (Pfam-B_61649 and Pfam-B_4314, respectively) that are present in several C-type lectin related proteins and thought to be involved in carbohydrate binding. Intact homologues of ORF 006 and 007 in the NAV strain are lacking due to five and seven base insertions at nucleotide 51 and 123, respectively. Of the other orthopoxvirus species so far examined, both of these ORFs are conserved in CPXV-GRI, and ORF 007 is also present in CPXV-BR. ORFs 006 and 007 may be a disruption of a larger gene as they align with the 5′ and 3′ regions, respectively, of the fowlpox virus (FWPV-FCV) ORF 238 (Accession No. AF 198100). A third gene coding for a lectin-like protein might have existed in a progenitor of ECTV-MOS, since VACV-COP ORF A40R can be aligned with a three frame translation of Region S (Table 1). The A40R protein has low but significant aa homology to natural killer cell proteins that recognize class I MHC antigens, and a role in pathogenesis has been shown in the VACV-WR mouse dermal, but not the intranasal, infection models (Tscharke et al., 2002; Wilcock et al., 1999). If A40R is indeed involved with inhibition of natural killer cell activity, it is interesting that a virus such as ECTV, which is orders of magnitude more virulent for the mouse than VACV, would lack such a potentially important molecule.

Viral membrane proteins

ECTV-MOS encodes 35 putative membrane proteins (Table 2). By analogy to VACV-COP ~14 and 5 membrane proteins are associated with IMV and EEV, respectively,

with an additional protein (ORF 137.5) transiently associated with IEV. Since only 20 of the 35 putative membrane proteins are involved in virion formation, at least some of the remaining proteins may have alternative functions. ECTV-MOS ORF 138 is an example of a putative membrane protein with a potentially important role in the natural virus life cycle in the animal host. ECTV-MOS ORF 138 (VACV-COP A38L homologue) contains an N-terminal immunoglobulin superfamily domain, followed by five membrane-spanning domains, and a short cytoplasmic tail. ECTV-MOS ORF 138, similar to all examined orthopoxviruses to date, has low but significant homology with CD47 across its entire predicted protein sequence (~29% identity versus mouse CD47). CD47, also called integrin-associated protein (IAP), is expressed on all cell types. CD47 is a receptor for thrombospondin family members, is a component of supramolecular complexes containing specific integrins, and interacts with signal regulatory protein (SIRP)α (Brown and Frazier, 2001). By analogy to CD47, the orthopoxvirus ortholog may have the capacity to modulate a wide range of integrin ligation events including cellular adhesion, migration, and diapedesis, and perhaps engage an inhibitory receptor such as SIRP, which is found on neurons and myeloid cells (Ravetch and Lanier, 2000). Previous studies with VACV-WR found that the A38L protein was present in low abundance in infected cells and was not detectable in IMV or EEV (Parkinson et al., 1995). Furthermore, A38L was not found to contribute to production of IMV and EEV, plaque size, and virulence in the intranasal mouse model; however, its overexpression in tissue culture correlated with Ca^{2+} entry into, and necrosis of, infected cells (Parkinson et al., 1995; Sanderson et al., 1996). These results were surprising in light of our increasing understanding of CD47 biology, but may reflect the fact that VACV is not a natural pathogen of the mouse, the intranasal route of inoculation may not be optimal for observing A38L function, or the parameters (weight loss and signs of illness) used to assess A38L function in the mouse lacked sufficient sensitivity to measure an effect.

TNF receptor-protein family

The TNF receptor protein family contains the largest number of distinct orthopoxvirus immunomodulators of any protein family. CPXV encodes four different binding proteins for TNF (CrmB, CrmC, CrmD, and CrmE) each with different patterns of expression and/or different affinities for TNF-α and TNF-β (Hu et al., 1994; Loparev et al., 1998; Saraiva and Alcami, 2001; Smith et al., 1996). In ECTV-MOS the CrmB homologue is missing, and the CrmC (Region V) and CrmE homologues (Region B/BA) are fragmented. The only ORF that appears to express a functional TNF binding protein (vTNFbp) is ORF 003/170 (CrmD) (Chen et al., 2000; Loparev et al., 1998). ORF 003/170 has transcription regulation signals consistent with early and late expression during the infection cycle; however, the binding specificity of its vTNFbp/CrmD product remains unclear. Surface plasmon resonance measurements indicated that vTNFbp/CrmD bound to immobilized human and rat TNF-α as well as human TNF-β (Loparev et al., 1998). In the same study these findings were supported by functional assays which indicated that vTNFbp/CrmD inhibited TNF-α and TNF-β cytotoxicity; however, others using a filter-based binding assay found vTNFbp/CrmD bound mouse, human, and rat TNF-α, but not human TNF-β. Full-length and presumably functional CrmD homologues have been observed in genomes of ECTV strains MP-3, MP-4, and Munich-SF. ORF 008 is related to the CrmB family of poxvirus genes, has an ortholog only in CPXV (>90% identical), and is predicted to be a secreted protein. The amino-terminal 35 aa sequence has no significant match with the nonpoxvirus protein database, whereas there is a 42% identity with CPXV-BR Crm B over the remaining 167 residues (83%) of the protein sequence. Recently CPXV and ECTV, but not VARV, VACV, MPXV, and CMLV, have been shown to encode a fifth functional member of this family, a secreted protein with homology to the receptor-binding domain of mouse and human CD30 (Panus et al., 2002; Saraiva et al., 2002). A CD153 binding protein (vCD30) was shown to bind specifically and with high affinity to the CD30 ligand, CD153, completely blocking CD30/CD153 interaction. Because CD153 is expressed on activated T cells, monocytes, macrophages, eosinophils, and neutrophils, and CD30 is likewise abundant on inflammatory/immune cells, production of vCD30 at the site of ECTV infection in mouse skin may be important for disruption of the initial innate response or its amplification.

Host range proteins

Although experimental infections of a wide range of small animal models has indicated that ECTV probably has a very narrow host range, infecting one or a small number of rodent species, the natural animal reservoir has yet to be determined. The wild mouse, *Mus musculus*, a possible candidate as infections of laboratory raised outbred *M. musculus*, which were derived from feral mouse populations in the Eastern USA and the former Czechoslovakia, showed a pattern of disease resistance/susceptibility consistent with a natural host for ECTV (Buller et al., 1986). This may also explain the origins of the now well-known resistance/susceptibility disease patterns of inbred strains of laboratory mice, which are mosaics of the genomes of European and Asian *M. musculus* subspecies (Bonhomme, 1986; Wallace and Buller, 1985; Yonekawa et al., 1982). In the broadest context, virus host range in nature is determined by a summation of activities of viral genes that allow efficient virus replication, spread within the animal, and transmission in the face of inflammatory and immune host defense mechanisms. Here we consider host range in a narrower sense to mean virus functions that affect virus replication and release from specific cell types.

Table 3
Predicted ectromelia virus host range proteins

ORF	Predicted MW kDa	Other name/motif	Orthologs	References
012	28.4	$_{P}$28/zinc finger	BSH-D6R	Senkevich et al., 1994, 1995; Upton et al., 1994; Brick et al., 1998, 2000
015	18.1	Ankyrin repeats	COP-C7L	Perkus et al., 1990; Chen et al., 1993
022	32.7	Ankyrin repeats	COP-KIL	Gillard et al., 1986; Chen et al., 1993
043	21.6	$_{P}$20/$_{P}$25 dsRNA binding	COP-E3L	Chang et al., 1992; Beattie et al., 1996; Langland et al., 2002; Smith and Alcami, 2002b
168	42.5	SPI-1/Serpin RSL	COP-C12L	Kotwal and Moss, 1989; Moon et al., 1999

The following orthopoxvirus genes have been shown to be involved in host range in established cell lines or primary cultures: CHOhr (CPVX-BR 025), K1L (VACV-COP K1L), C7L (VACV-COP C7L), p28 (RING zinc-finger, ORF 012), SPI-1 (RPV-UTR 005), E3L (VACV-COP E3L), and K3L (VACV-COP K3L) (Table 3). In the ECTV-MOS genome, we identified predicted homologues for all but the CHOhr gene (Region F) and VACV K3L (Region M). The cell lineage tropisms governed by the CHOhr gene, K1L, and C7L genes have been well-described (Drillien et al., 1978, 1981; Gillard et al., 1985; Perkus et al., 1990; Spehner et al., 1988). Although these predicted proteins are not significantly related (except for the presence of ankyrin repeats), C7L, K1L, and CHOhr proteins in tested porcine or human lines, and K1L and CHOhr proteins in rabbit RK-13 cells, are functionally interchangeable. In tissue culture, the CHOhr gene supported orthopoxvirus replication in the broadest range of cell types tested and was shown to delay but not block apoptosis (Ink et al., 1995), yet it is not conserved among orthopoxviruses as the gene is fragmented in ECTV, VARV, VACV, CMLV, and MPXV. ECTV's narrow host range in nature is not solely due to the lack of a functional CHOhr gene since ECTV-MOS mutants with a repaired CHOhr gene had an expanded capacity for replication in rabbit and hamster-derived tissue culture lines, yet similar to ECTV-MOS (wild-type) wt, still failed to replicate in the skin of the Syrian hamster (Chen et al., 1992) (M. Buller, unpublished data). Both ORF 015 and ORF 022 have 95 and 97% identity at the aa level with VACV-COP homologues C7L and K1L, respectively, and thus would be expected to functionally interchange with their VACV homologues; however ORF 022 could not functionally substitute for K1L and support optimal VACV replication in rabbit-derived RK-13 and SIRC cell lines (Chen et al., 1993). Infections of RK-13 cells with ECTV-MOS and VACV mutants lacking an intact K1L gene resulted in transient expression of early genes followed by rapid and irreversible cessation of both virus and host protein synthesis compatible with the onset of apoptosis. In addition, footpad infections of the disease-susceptible ANCR or -resistant C57BL/6NCR mice with a ORF 022-lacking ECTV-MOS yielded a pathogenesis pattern indistinguishable from wt, suggesting that the K1L host-range homologue is not important for ECTV-MOS in vivo tissue replication, spread, and lethality under the tested set of experimental conditions.

An intact p28 gene has been identified in a majority of orthopoxvirus species and representatives of *leporipoxvirus*, *yatapoxvirus*, and *capripoxvirus* genera, although it is disrupted or deleted in the WR, COP, MVA, and TIA strains of VACV. The prominent feature of the protein is a C-terminal RING zinc-finger that is minimally defined by a configuration of seven Cys (C) and His (H) residues necessary for chelation of two zinc ions (C_3HC_4) (Freemont et al., 1991). Although a large number of proteins with diverse functions have a RING zinc-finger motif, the spacing between the signature residues and the overall aa composition of proteins shows considerable variation. The ECTV-MOS p28 was shown to have significant homology to the RING zinc-finger region of proteins of unknown function from vertebrates and invertebrates, which form the makorin protein family (Gray et al., 2000). Studies with ECTV-MOS p28$^-$ virus in cell cultures and ANCR mice have suggested that p28 has a very important role in the natural life cycle of ECTV. Following footpad inoculation, the LD_{50} of p28$^-$ virus in ANCR mice is at least a million-fold higher, and the infectivity burden in liver and spleen is at least a thousand-fold lower, than wt virus (Senkevich et al., 1994). This attenuation of ECTV-MOS pathogenicity in ANCR mice can be explained by a failure of the virus to replicate in macrophage lineage cells at successive steps in the spread of virus from the skin to liver (Senkevich et al., 1995). The diminished replication of p28$^-$ virus in macrophages from ANCR mice may result from inefficient suppression of apoptosis as p28 has been shown necessary to protect ECTV-MOS-infected HeLa cells from UV-induced apoptosis (Brick et al., 2000).

SPI-1 is a closely related member (46% identity in ECTV-MOS) of a serpin (serine proteinase inhibitors) superfamily whose members act as suicide substrates for serine and selected cysteine proteinases. The specificity of the serpin is determined by the reactive-site loop (RSL), which is designated by aa P15 to P5′. The RSL interacts directly with the target proteinase, and proteolysis occurs at the scissile bond between residues P1 and P1′, yielding a long-lived complex. SPI-1 has been shown specifically to interact with cathepsin G (Moon et al., 1999) and has been

shown necessary for optimal host-range in vitro (A549 and PK-15), whereas its role in vivo is less clear (Ali et al., 1994; Kettle et al., 1995; Thompson et al., 1993). SPI-1 has been shown to affect apoptosis in RPV-UTR-infected A549 and PK-15 cells and may do so in a caspase-independent manner (Brooks et al., 1995; Moon et al., 1999). ECTV-MOS SPI-1 (ORF 168) showed >95% identity with VACV-COP homologues. The ECTV-MOS and RPV-UTR SPI-1 homologues share an identical RSL, and therefore, perhaps share an identical function.

E3L and K3L contribute to the host range of orthopoxviruses by blocking endogenous antiviral pathways that are enhanced by interferons (IFN). E3L acts as an inhibitor of the dsRNA-dependent protein kinase (PKR) and 2′-5′-oligoadenylate synthetase (OAS) pathways by binding and sequestering dsRNA activator molecules (Langland and Jacobs, 2002). Activation of PKR by viral infection in the absence of E3L resulted in apoptosis as did overexpression of active PKR in the absence of infection (Lee and Esteban, 1994). K3L has partial homology to the amino-terminal region of eIF2, inhibits autophosphorylation of PKR and eIF2α substrate phosphorylation, and is thought to act as a competitive inhibitor of PKR (Carroll et al., 1993; Davies et al., 1993). A recent study by Langland and Jacobs using VACV infections of BHK and HeLa cells found that the importance of E3L and/or K3L for replication varied between the two cell types according to virus RNA levels, endogenous PKR activity, and the presence of a functional OAS pathway (Langland and Jacobs, 2002). By extrapolating these findings to ECTV, one would predict that the PKR and OAS pathways would not be activated and exert an antiviral effect, if the major cell type(s) important in maintaining the virus life cycle failed to produce dsRNA that exceeded the sequestering capacity of the ECTV E3L homologue (ORF 043). If this model was correct, there would be no selection pressure to maintain a functional K3L gene. Consistent with this hypothesis, the K3L homologue appeared nonfunctional in the ECTV strains sequenced to date. As compared to the VACV-COP K3L gene, the ECTV-MOS homologue has a one-base insertion after base 93 and a two-base deletion following base 174, which resulted in a three-frame translation of ECTV-MOS DNA corresponding to Region M (Beattie et al., 1991). Interestingly MPXV-ZAI also lacks an intact homologue of K3L, and the E3L homologue has an amino terminal deletion. If K3L and E3L homologues were solely responsible for protecting orthopoxvirus replication from the antiviral effects of IFNs, one might expect that replication of VACV and ECTV in identical cell lines pretreated with IFNs might be similar, or VACV replication might be more resistant due to the presence of a functional K3L gene; however, this is not the case (Karupiah et al., 1993a). In mouse L929 cells the replication of a large number of isolates of ECTV was shown to be resistant to mouse IFN-α/β and IFN-γ, whereas the VACV-WR was sensitive (Smith and Alcami, 2002b).

ECTV attenuation of the innate response following infection

In the early stage of infection, ECTV-MOS host response modifiers (HRMs, Table 4) may restrict the spread of inflammatory cytokines in the epidermis and may block intracellular responses to infection that include the following; 1) induction of apoptosis; 2) further secretion of inflammatory/immune mediators; 3) synthesis or activation of intracellular antiviral pathways; and 4) extracellular matrix or cellular interactions via cell surface molecules.

ECTV-MOS HRMs target both primary and secondary epidermal inflammatory cytokines/chemokines. vIL-1βbp, vIL-18bp, and SPI-2/CRMA inhibit the activity of primary inflammatory cytokines (Table 4). In addition, ORF 145 (VACV-COP ORF A46R) may further inhibit IL-1 signaling at a stage between receptor engagement and the Myd88 adaptor molecule (Bowie et al., 2000). In view of this redundant targeting of the IL-1 type IR, IL-18R, and Toll-like receptors (TLR) common MyD88/Traf 6/NF-κB signal transduction pathway, it was unexpected to find that the ECTV-MOS homologue of VACV-COP A52R was disrupted (Table 1, Region U). COP ORF A52R is a broad and potent inhibitor of NFκB activation by IL-1, IL-18R, and multiple Toll-like receptors TLR (Harte et al., 2003). As compared to COP A52R, ECTV strains MOS and NAV homologues have an effective 29 and 30-bp deletion, respectively, commencing 73 nucleotides downstream from the AUG start codon. Providing a second initiation codon is recognized in the MOS homologue, it is possible that a 106-aa protein could be made which would be homologous with COP A52R aa 33 to 126.

Secondary epidermal cytokines/chemokines: IFN-α (but not IFN-β), IFN-γ, TNF-α, and CC chemokines are each targeted by a specific binding protein (Table 4). By analogy to VACV, vIFN-α/βbp may be found on cell surfaces of infected and uninfected cells as well as in a soluble form, suggesting a role for blocking IFN-α autocrine and paracrine functions (Colamonici et al., 1995). vIFN-α/βbp may help break the linkage between the innate and adaptive immune response by inhibiting the interaction of IFN-α with LC (Kaser and Tilg, 2001). An important role of vIFN-α/βbp in orthopoxvirus pathogenesis was supported by studies that found inactivation of VACV-WR vIFN-α/βbp homologue resulted in virus attenuation by the intranasal and intracranial routes of inoculation in mice (Colamonici et al., 1995; Symons et al., 1995). The in vivo role of the vTNFbp has been studied most thoroughly in MYXV-LAU infections of rabbits where a knockout virus showed a marked decrease in pathogenicity (Upton et al., 1991). TNF function may also be targeted downstream from receptor engagement in at least two places. TNF signal transduction and that of the IL-1 type IR, IL-18R, and TLR may be further attenuated by an as yet to be identified virus gene product(s) that affects NF-κB activation by interfering with IκBα degradation as described for CPXV (Oie and

Table 4
Predicted ectromelia virus innate immune host response modifiers

ORF	Predicted MW (kDa)	Motifs/Proposed function	Ortholog	References
		Blockers of interferon		
043	21.6	Inhibits PKR and OAS	COP-E3L	Chang et al., 1992; Beattie et al., 1996; Langland and Jacobs, 2002; Smith and Alcami, 2002b
083	19.7	Blocks IFN-γ signal transduction	COP-H1L	Najarro et al., 2001
158	30.5	vIFN-γbp	COP-B8R	Upton et al., 1992; Mossman et al., 1995; Smith and Alcami, 2002b
166	41.0	vIFN-α/βbp	COP-B19	Symons et al., 1995; Colamonici et al., 1995; Smith and Alcami, 2002b
		Cytokine/chemokine binding proteins		
001/172	26.7	vCC-CKbp (T1/35-kDa)	COP-C23L	Smith et al., 1997; Graham et al., 1997; Smith and Alcami, 2000a
003/170	35.1	vTNFbp/CrmD	BR-211	Smith et al., 1997; Loparev et al., 1998; Smith and Alcami, 2000a
009	12.2	CD153bp (vCD30)	BR-014	Panus et al., 2002; Saraiva et al., 2002
013	15.8	vIL-18bp	BSH-D7L	Novick et al., 1999; Born et al., 2000; Calderara et al., 2001; Symons et al., 2002a
161	38.6	SP1-2/CrmA	COP-B14R	Palumbo et al., 1989; Ray et al., 1992; Turner et al., 2000
163	37.5	vIL-1βbp	COP-B16R	Spriggs et al., 1992; Alcami and Smith, 1992; Smith and Alcami, 2000a
		Complement inhibitor		
017	28.6	Sec complement bp (VCP/EMICE)	COP-C3L	Kotwal and Moss, 1988; Isaacs et al., 1992
		Specific mechanism of action unknown		
008	22.4	TNFR-C terminal region	BR-013	
019	14.1	Cytoplasmic protein	COP-N1L	Kotwal et al., 1989; Bartlett et al., 2002
138	31.5	CD47-like	COP-A38L	Parkinson et al., 1995; Sanderson et al., 1996
139	45.4	Secreted semaphorin	COP-A39R	Comeau et al., 1998; Gardner et al., 2001
140	25.4	Attenuates inflammatory cell influx	COP-A41L	Ng et al., 2001
143	39.3	Hydroxysteroid dehydrogenase	COP-A44L	Moore and Smith, 1992; Sroller et al., 1998
144	13.7	Superoxide dismutase-1	COP-A45R	Almazan et al., 2001
145	27.6	Attenuates IL-1 activation of NFkappaB	COP-A46R	Bowie et al., 2000

Pickup, 2001). Also SPI-2/CrmA was shown to block the activity of caspase 1, 8, and 10, thereby inhibiting the induction of apoptosis induced by a variety of different stimuli including TNF and growth factor deprivation (Ray et al., 1992; Ray and Pickup, 1996; Turner et al., 2000). SPI-1 and SPI-2/CrmA was shown to attenuate apoptosis induced via the Fas receptor by alloreactive CTL, but did not protect virus-infected cells from virus-specific CTL, which mainly kill targets by the granule exocytosis pathway (Macen et al., 1996; Mullbacher et al., 1999; Wallich et al., 2001). The viral CC chemokine binding protein (vCC-CKbp; ORF 001/172) is the most abundant of the ECTV-MOS-secreted proteins (Chen et al., 2000). vCC-CKbp does not bind CXC, C, and CX3C chemokines. vCC-CKbp was shown to block a large number of CC chemokines including MCP-1, a major product of keratinocytes and a chemoattractant for monocytes (Burns et al., 2002). Thus the CXC chemokine, IL-8, which is a major product of activated keratinocytes and fibroblasts and a chemoattractant for neutrophils, should not be affected by ECTV infection unless there is an as yet to be discovered virus-encoded modulator of IL-8 function. By analogy to MYXV, the vCC-CKbp may interact with glycosaminoglycans that are found on the exterior surface of cells and within the extracellular matrix, thereby maintaining a high concentration of protein proximal to the focus of infection, preventing the migration of inflammatory cells into the virus lesion (Seet et al., 2001). Inactivation of the vCC-CKbp gene in MYXV-LAU and rabbitpox virus strain Utrecht resulted in early leukocyte infiltration into tissue sites of virus infection, but no effect on virus lethality (Graham et al., 1997; Lalani et al., 1999; Martinez-Pomares et al., 1995).

We propose the action of ECTV HRMs in the epidermis results in a lower concentration of primary and secondary cytokines reaching the dermis, and a failure to generate optimal gradients of chemokines (especially CC chemo-

kines) to direct first inflammatory cells and then immune T cells to site of infection. The presence of replicating virus in the dermis, and the additional production of HRMs, would further restrict the activation of the endothelium to permit the diapedesis of inflammatory cells into the primary lesion. In addition the cytokine secretion pattern of dermal macrophages may be affected by a virus-encoded, secreted member of the semaphorin family, ORF 139, which was shown to induce monocyte aggregation through ICAM-1 and to secrete IL-6 and IL-8 (Comeau et al., 1998). Also vIL-18bp may hinder dermal macrophage-mediated activation of NK cells if present. Consistent with this hypothesis, peritoneal exudate cells from mice infected with an ECTV-MOS mutant lacking vIL-18bp showed reduced NK lytic activity and increased IFN-γ secretion as compared to wt (Born et al., 2000). Similar results were obtained in the VACV-WR intranasal mouse model (Symons et al., 2002a). vIFN-γbp may also be important in sequestering IFN-γ, the major antiviral cytokine produced by activated NK cells, and also a product of activated immune $CD4^+$ Th1 and $CD8^+$ T cells. Endogenous IFN-γ production in ECTV-MOS-infected C57BL/6 mice was shown critical for recovery from infection, in part by inducing the production of inducible nitric oxide synthase, and by analogy to VACV perhaps the chemokine MuMig (Karupiah et al., 1993a,b, 1998; Mahalingam et al., 1999). The in vivo importance of poxvirus vIFN-γbps has been examined using knockout viruses in several model systems, but the results obtained seem dependent on the virus, route of infection, host, and the binding specificity of vIFN-γbp (Mossman et al., 1996; Symons et al., 2002b; Verardi et al., 2001). With an ECTV-MOS mutant lacking vIFN-γbp virulence was unaffected following footpad infection of inbred and outbred mice (M. Buller, unpublished data). This result may indicate that other virus genes are able to compensate for the lack of vIFN-γbp. For example, ORF 083 may also target IFN-γ signal transduction (Najarro et al., 2001).

Activation of complement in the epidermis/dermis is likely inhibited by homologues of vaccinia complement protein (VCP). VCP inhibits both the alternative and the classical pathways of complement activation by binding and inhibiting C3b and C4b function and thereby reduces the production of inflammatory mediators C3a, C4a, and C5a (Kotwal et al., 1990; Kotwal and Moss, 1988). The ECTV-MOS homologue of VCP is encoded by ORF 017 (Table 4) and is also called ectromelia inhibitor of complement enzymes (EMICE) by analogy to the VARV homologue SPICE (Rosengard et al., 2002). EMICE, similar to VCP and SPICE, contains four complement control protein [CCP; also called short consensus repeat] (SCR) modules in common with members of the family of regulators of complement activation (RCA), and contains putative heparin binding sites that would facilitate docking on cell surfaces and perhaps virions to inhibit complement activation (A1 Mohanna et al., 2001; Smith et al., 2000b). EMICE is highly homologous with SPICE and VCP in CCP 2, CCP 3 and CCP 4 differing by only 16 aa overall; however, EMICE differs in CCP 1 from both SPICE and VCP by an identical 12 aa (including a contiguous 2 aa deletion). The significance of these aa differences in EMICE CCP 1 on the capacity to block activation of mouse complement awaits elucidation.

The ECTV HRMs are proposed to attack all of the major innate responses to infection in the skin at multiple points and may contribute to the final character of the developing immune response. A likely effect of the HRMs is the inhibition of recruitment of inflammatory and immune cells to the infected tissues, which extends the time for virus replication, shedding of virions into the environment, and maximizes the probability of virus transmission. To date there have been no orthopoxvirus HRMs identified that block the generation of immune T cell responses. On the contrary, orthopoxviruses are usually associated with the generation of long-lived immunity on recovery from infection. VACV is used both as a live vaccine for smallpox and as a vector to identify B and T cell epitopes on proteins from other pathogens (Moss, 1996; Paoletti, 1996). The failure of orthopoxviruses to evolve efficient inhibitors of the generation and function of immune T cells allows for a rapid containment of systemic infections. Providing the immune response can be developed in sufficient time to limit tissue destruction of vital internal organs, the host survives and transmission of progeny virus continues from the primary site of infection as in the case of ECTV infection of the resistant C57BL/6 mouse.

Other proteins

ECTV-MOS ORF 025 is a homologue of VACV-COP ORF F1L. ORF 025 may or may not be functional as it contains 30 copies of an eight aa (DNGIVQDI) repeat element very close to the N-terminus of the protein. VACV-COP ORF F1L and CPXV-GRI ORF G1L contain only one copy of this aa element but the ortholog in CPXV-BR (ORF 048) contains three copies of this repeat element. In other viruses, there are repeats of a shorter sequence (DDI); ECTV-NAV has one copy of the DDI element and 25 copies of DNGIVQDI. Interestingly, the aa sequence also varies between the closely related strains of CPXV, CMLV, and VARV. A number of immunomodulator proteins probably remain to be characterized as six putative secretory proteins still lack known functions (ORFs 007, 008, 063, 123, 130, and 140).

Phylogeny

There appears to be little genetic diversity in the ECTV species as virus isolates from world-wide outbreaks of mousepox have been found to be serologically indistinguishable from the original Hampstead strain (Fenner, 1982). In addition, restriction endonuclease analysis of genomic DNA from Hampstead and MOS strains revealed

an almost identical pattern of fragments following digestion with restriction endonucleases Hind III and Xho I (Mackett and Archard, 1979). Now a more comprehensive view of the genetic diversity of ECTV strains can be obtained by direct comparison of the genomic sequence of the MOS isolate with the genomic sequence of the NAV isolate. This second strain was designated as NAV as it was isolated from commercial, pooled mouse sera, which was the source of a 1995 outbreak of mousepox in a laboratory mouse colony at the Naval Medical Research Institute, Bethesda, MD (Dick et al., 1996).

Despite the fact that the isolation of the MOS and NAV strains was separated both temporally and geographically, there is very little DNA sequence variation that would be expected to have a biological consequence. The genomes of ECTV-MOS and -NAV strains exhibit a 99.5% nucleotide identity over their length (excluding the ITR regions). This is similar to values obtained for strains within the VARV species: 99.3% identity between VARV-BSH and VARV-IND and 99.1% identity between a VARV-BSH (major strain) and VARV-Garcia (minor strain). An interspecies comparison of ECTV-MOS, with VACV-COP, VARV-BSH, and CPXV yields 92, 89, and 90% nucleotide identity, respectively, reflecting the on average, 90% nucleotide sequence identity for cross-species nucleotide differences within the *Orthopoxvirus* genus. For comparison, capripoxvirus species, sheeppox virus, and goatpox virus exhibited 96% nucleotide identity, and strains within the species showed greater than 99.7% nucleotide identity (Tulman et al., 2002). The simplest explanation for the apparent lack of diversity among strains of a species from two distantly related genera is the fidelity of the poxvirus DNA polymerase. Most differences between the MOS and NAV genomes are located within or close to the ITRs. Compared to the MOS strain, the NAV strain has two deletions of approximately 650 and 2220 bp starting from nucleotides 4607 and 7312, respectively. Both of these deletions are in regions of fragmented genes in both genomes, Regions A and D in strain MOS (Fig. 2 and Table 1). In addition to the small number of differences between the strains that have been mentioned above, there are two possibly more significant changes to predicted ORFs. The phospholipase D-like gene (VACV-COP ORF K4L) is present in strain NAV, but disrupted in strain MOS by a four nucleotide deletion at nucleotide 556 of the gene that results in a frameshift and premature stop codon after 185 aa (Region N). The predicted superoxide dismutase-like protein (ORF 144) is truncated in strain NAV by approximately 30 of 125 aa; it is not clear if this affects the function of the protein because none of the chordopoxvirus proteins have been shown to have enzymatic activity.

Figure 3 demonstrates the predicted phylogenetic relationships between ECTV-MOS and other viruses in the *Poxviridae* family. The phylogenetic inferences in Fig. 3A and B represent a composite of the alignments of 40 different proteins that were conserved among all of the *Poxviridae* species with available genomic sequences. Fig. 3A shows the predicted evolutionary relationship of representatives of nine different genera, while Fig. 3B displays the predicted relationship between species only within the *Orthopoxvirus* genus. Figs. 3C and D show the predicted phylogeny based upon nucleic acid sequence alignments of the coding sequences representing either the gene for DNA polymerase (VACV gene E9L) or the gene for nucleoside triphosphatase (VACV gene D5R). Branch lengths of the evolutionary trees are proportional to the number of aa or nucleotide sequence changes between any two nodes (see scale at the bottom of each panel). Bootstrap resampling confidence values based upon 1000 replicates are displayed along each branching point on the trees. The tight grouping within the *Orthopoxvirus* genus shown in Fig. 3A demonstrates the close phylogenetic relationship among these orthopoxvirus species in comparison to all other *Poxviridae* genera. Within the orthopoxviruses, ECTV in general shows the greatest phylogenetic distance compared with all other orthopoxvirus species, with CPXV displaying the next most distant set of sequences. The branching order relationships among the orthopoxvirus species are all essentially the same when comparing either the combined set of 40 different protein gene alignments (Fig. 3B) or the individual nucleotide gene alignments (Figs. 3C and D). Minor branching differences between VACV-COP and MPXV-ZAI in Figs. 3C and D are probably not significant. While ECTV appears to show more evolutionary distance from other orthopoxvirus species, the distances are not great enough to provide any confidence in trying to root the tree at any particular place.

Therefore, based on protein and nucleotide sequence divergence no conclusions can be drawn as to the ancestral relationships between these sequences, and no temporal direction of evolutionary divergence can be inferred. As an alternative, it might be possible to examine the genome complement of apparently intact ORFs conserved between orthopoxvirus species as a means of inferring ancestral relationships among these genomes. When comparing conserved gene sets between all of the orthopoxvirus species, CPXV contains the most inclusive set of orthopoxvirus genes (data not shown). That is, most of the genes present in all of the other orthopoxvirus genomes are also present in CPXV. None of the other orthopoxvirus species sequenced to date contain such a diverse “superset” of conserved genes similar to that contained within CPXV. While this analysis is not definitive (it is highly dependent on the predicted gene set for each genome, and we used a fairly conservative set of predictions for this analysis), it does support the previously stated hypothesis that a CPX-like virus may be the ancestor of all modern-day orthopoxvirus species (Shchelkunov et al., 1998).

Materials And methods

Cells and viruses

The MOS strain of ECTV was isolated in the laboratory of Professor V.D. Soloviev in Moscow. In 1946 Dr. C.H. Andrewes sent the MOS strain to Dr. Frank Fenner, who over the years distributed the strain to a large number of investigators. A plaque-purified isolate of ECTV-MOS (ATCC VR-1374), designated Mos-3-P2, was propagated in an African green monkey kidney cell line, BS-C-1 (ATCC CCL 26) (Chen et al., 1992). Cells were grown in Eagle's minimum essential medium (MEM) containing 10% FETALCLONE II (Hyclone, Logan, UT), 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). Virus infectivity was estimated as described previously (Chen et al., 1992).

Purification of ectromelia virus genomic DNA

Five cultures containing 2×10^7 BS-C-1 cells were infected with ECTV-MOS at an m.o.i. of 1–5 PFU/cell in 5 ml of Dulbecco's modified Eagle's medium (DMEM, Bio-Whittaker, Walkersville, MD) containing 2% FETAL-CLONE II (DMEM-2). After 1 h at 37°C cultures were supplemented with a further 10 ml of prewarmed DMEM-2 and incubated until maximum cytopathic effect. Cells were scraped into the culture supernatant and centrifuged at 850 *g* for 5 min in a microcentrifuge. The cell pellet was resuspended in a total of 2.4 ml phosphate-buffered saline and divided equally among four 1.5-ml microfuge tubes. The cell suspension was frozen and thawed three times and mixed with 600 μl of 1% Triton X-100, 40 mM EDTA, and 68 mM β-mercaptoethanol. A post 850 *g* supernatant containing virions was centrifuged at 2100 *g* for 15 min in a microcentrifuge. The viral DNA was extracted from virions by SDS–proteinase K treatment followed by phenol-chloroform as described before (Moss and Earl, 1998). DNA was resuspended in ddH_2O and concentration was estimated by agarose gel electrophoresis.

Cloning and sequencing of an ectromelia virus heterogeneous G/C-rich region

An ECTV-MOS fragment of ~700 bp containing G/C-rich region was PCR-amplified by using primers EVGCR-5′ (5′-GCGCTCGAGGTTATTTATAGCA-GATATAG-3′) and EVGCR-3′ (5′-GCGGGATCC-CACTAAGAGATTATTTTACC-3′). After digestion with *Bam*HI and *Xho*I, this fragment was cloned into similarly digested pGEM-7Zf(+) (Promega, Madison, WI). Ten clones were selected, and their plasmid DNAs were sequenced as described.

Estimation of the size of the direct-repeat-containing region by gel electrophoresis

The direct-repeat-containing region was PCR-amplified using primers LE-1.25R (5′-GGACGTTATTGTTTAA-GAAATAG-3′) and LE-2.05L (5′-CAAGTGTGATAC-GATTATGAG-3′). By agarose gel electrophoresis the size of this fragment was calculated to be 962 bp using a 100-bp DNA ladder as size markers (Life Technologies, Inc., Rockville, MD). This agrees favorably with the 963-bp size based on the assembled sequence (including nonrepeat primer sequences). There are 10.4 repeats, and each of them is 85 bp long.

DNA sequencing

With the exception of the terminal hairpin loops and the right-hand end of about 33.5 kb (32 kb of which was sequenced before) (Chen et al., 2000), the entire ECTV-MOS genome was divided into 16 overlapping fragments of about 11 kb (J. Esposito, personal communication). Each fragment was amplified from genomic DNA using Expand Long Template PCR System (Roche Diagnostics Corp., Indianapolis, IN) following the manufacturer's instructions. PCR products were purified using either ExoSAP-IT (USB Corp., Cleveland, OH) or QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA), if the product was less than 10 kb. If the desired PCR fragment was contaminated with additional products, the band of interest was gel-purified using QIAEX II and QIAquick Gel Extraction Kits for bands $>$ 10 and $<$10 kb, respectively. The left-hand end fragment of about 18 kb was sequenced by primer walking, the other 15 fragments were sequenced with a bank of sequencing primers, which were designed according to the sequence alignment of VARV-BSH and VACV-COP. Sequencing primers were designed to be about 450 bp apart to ensure adequate overlap. Gap closure was achieved by primer walking. Both strands of each fragment were sequenced. Sequencing reactions were carried out using CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter), and run on CEQ 2000XL DNA Analysis System (Beckman Coulter).

DNA sequence analysis

DNA sequences were assembled from the raw sequencing data using the Staden Package. Annotation of the genome was performed using Artemis and the Viral Genome Organizer (Upton et al., 2000) and analyzed with the EMBOSS Programs. The annotated genome was loaded into the Poxvirus Orthologous Clusters database, which is available from the Poxvirus Bioinformatics Resource Center (www.poxvirus.org) (Ehlers et al., 2002). Pairwise comparisons of whole genomes were performed using the programs WABA and MUMmer.

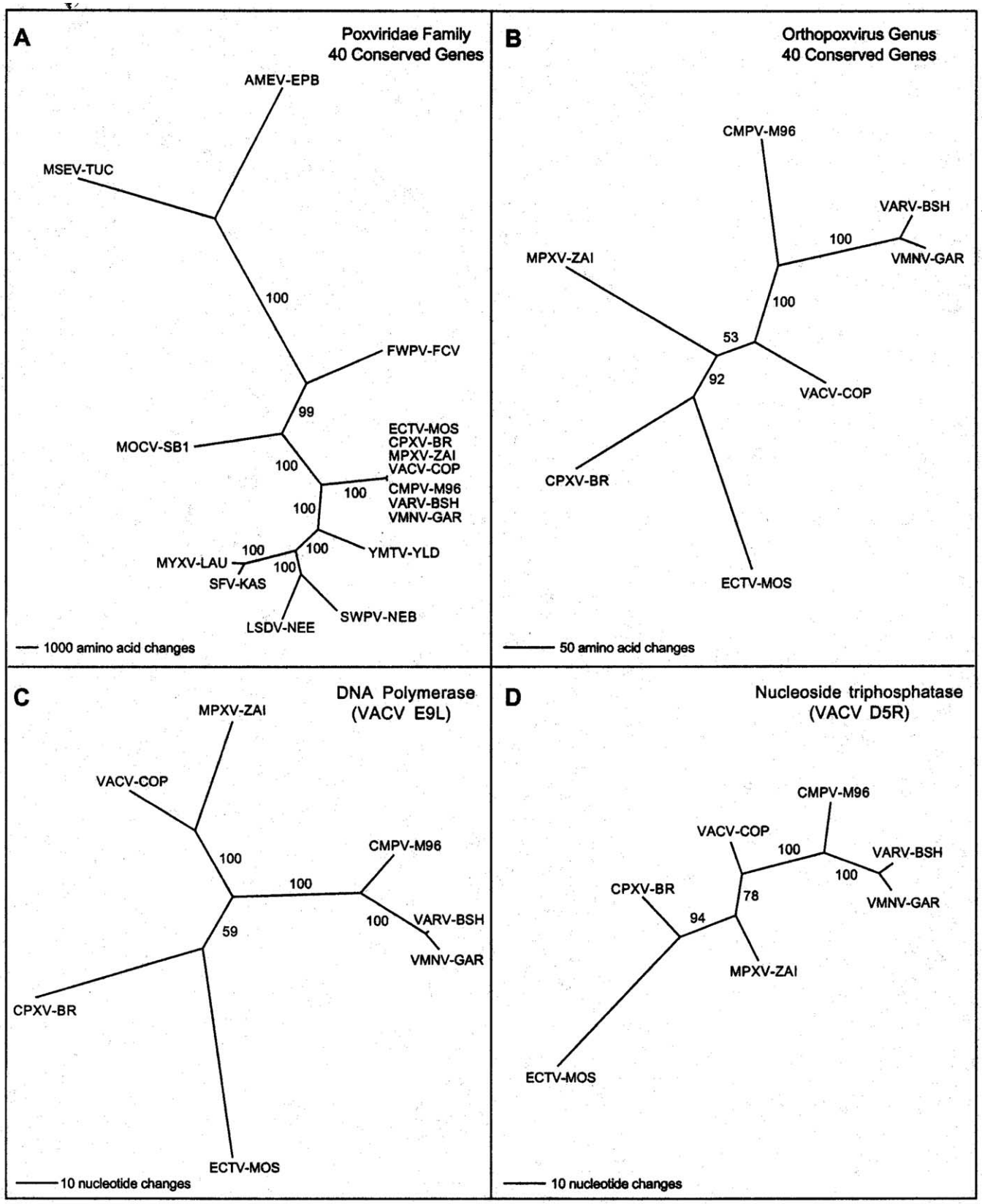

Fig. 3. Poxviridae phylogenetic predictions. A and B show phylogenetic predictions based upon amino acid alignments of 40 conserved protein coding sequences derived from the complete genomic sequences of either representative Poxviridae species (A) or representative orthopoxvirus species (B). C and D show phylogenetic predictions of aligned orthopoxvirus nucleotide sequences based on either the coding sequence of the DNA polymerase (VACV-COP E9L equivalent) gene (C) or the coding sequence of the nucleoside triphosphatase (VACV-COP D5R equivalent) gene (D). All predictions were calculated using the Branch-and-Bound search method with maximum parsimony as the optimality criterion. Bootstrap resampling confidence values on 1000 replicates are displayed at each branch point. Branch lengths are proportional to distance (the number of amino acid or nucleotide changes), and the distance scale for each prediction is given at the bottom of each panel.

Phylogenetic prediction

To predict the evolutionary relationship between ECTV and other Poxvirus genomes, we chose to analyze 40 different proteins that are conserved amongst 16 different poxvirus species representing nine different genera that have had their genomes completely sequenced. Each set of 40 proteins containing 16 different sequences was separately aligned using ClustalW. Prior to phylogenetic inference, each of the 40 alignments were concatenated together into one large contiguous alignment, and this alignment was then imported into the program suite PAUP* version 4.0b10. Evolutionary predictions were then calculated on the contiguous alignment of all 40 proteins from all of the 16 species; 40 proteins in a contiguous alignment from just the *Orthopoxvirus* genus (seven species) and the aligned nucleotide sequences of either the DNA polymerase gene or nucleoside triphosphatase gene from the seven orthopoxvirus species. Displayed are predictions that were made using the Branch-and-Bound search method with maximum parsimony as the optimality criterion.

Bootstrap resampling confidence values on 1000 replicates were calculated also using Branch-and-Bound with maximum parsimony. Evolutionary predictions, which were calculated using maximum likelihood as the optimality criterion or using Neighbor-joining clustering, produced trees with no significant difference when compared to those obtained with maximum parsimony. The Accession Nos. of the complete genomic sequences of the viruses used in this analysis are as follows: Amsacta moorei entomopoxvirus, AF250284; camelpox virus strain M96, AF438165; CPXV-BR, AF482758; ECTV-MOS, AF523264; Fowlpox virus strain FCV, AF198100; Lumpy skin disease virus strain Neethling, AF325528; Melanoplus sanguinipes entomopoxvirus, AF063866; Molluscum contagiosum virus, U60315; MPXV-ZAI, AF380138; Myxoma virus strain Lausanne, AF170726; Shope (rabbit) fibroma virus, AF170722; Swinepox virus isolate 17077-99, AF410153; VACV-COP, M35027; VARV-BSH, L22579; VARV minor strain Garcia, Y16780; Yaba-like disease virus, AJ293568. The identities of the 40 proteins used in the analysis along with the multiple sequence alignments are available as supplementary material at (www.poxvirus.org).

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