THE ANALYSIS OF THE COMPLETE GENOMES OF RABBITPOX VIRUS UTRECHT AND THREE WEST AFRICAN ISOLATES OF MONKEYPOX VIRUS

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ABSTRACT

The Orthopoxviruses (OPVs) comprise a group of viruses that possess very similar genomes; they vary considerably, however, in virulence. Among them, rabbitpox virus (RPXV) and monkeypox virus (MPXV) are the focus of this thesis. RPXV is closely related to vaccinia virus (VACV) but is significantly more virulent in rabbits. The West African isolates of MPXV, which also caused the human monkeypox 2003 outbreak in the USA, have different disease profiles from the Central African MPXV. To determine the basis for these differences, the complete genomes of RPXV-UTR and three West African isolates of MPXV were sequenced and analyzed.

The result of the RPXV study indicates that 3 RPXV genes, alone or in combination, likely play a key role in the enhanced RPXV-UTR virulence over VACV isolates. These genes encode: the RING finger protein (RPXV-UTR 008), an ankyrin repeat family protein (RPXV-UTR 180) and the chemokine binding protein (RPXV-UTR 001/184) in the inverted terminal repeats (ITR) of RPXV.

Examination of the evolutionary relationship between RPXV-UTR and other OPVs was carried out using the central DNA sequence of the genome that is conserved among all completely sequenced OPVs and also the protein sequences derived from the 49 genes present in all completely sequenced Chordopoxviruses (ChPV). The results of these analyses both confirm the hypothesis that RPXV-UTR is most similar to VACV.

An animal study found that the Central African MPXV isolate is more virulent than the West African MPXV isolate. The comparison of the three West African isolates MPXV-COP-58, MPXV-SL-V70, and MPXV-WRAIR, and the Congo basin (Central Africa) isolate MPXV-ZAI-96-I-16 shows that the MPXV-ZAI-96-I-16 ORF D14L, which encodes an inhibitor of human complement, is most likely the virulence gene responsible for the pathogenesis differences between the West and Central African isolates. These results explain the lack of fatalities in the 2003 MPXV outbreak in the USA, which was caused by importation of a West African MPXV isolate.

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List of Abbreviations

A, adenine

aa, amino acids

bp, base pair

ATCC, American Type Culture Collection

BBB, Base-By-Base software

BP, binding protein

BPSV, bovine papular stomatitis virus

BSC-40, cell line derived from African green monkey kidney

C, cytosine

CBP, chemokine binding protein

CCP, complement control protein

CHO, Chinese hamster ovary

ChPV, Chordopoxvirus, or Chordopoxvirinae

CMLV, camelpox virus (-M96, strain Kazakhstan; -CMS, strain CMS)

CNPV, canarypox virus

CPXV-BR, cowpox virus strain Brighton Red

Crm, cytokine response modifier

CRPs, complement regulatory proteins

CTLs, cytotoxic T lymphocytes

DAF, decay-accelerating factor

DMEM, Dulbecco's Modified Eagle's Medium

DNA, deoxyribonucleic acid

DR, direct repeat

DRC, Democratic Republic of the Congo

ds, double strand

dsRNA, double-strand ribonucleic acid

ECTV, ectromelia virus (-MOS, strain Moscow; -NAV, strain Naval)

DED, death-effecter domain

EEV, extracellular enveloped virus

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

eIF-2 , eukaryotic initiation factor 2

EMBOSS, European Molecular Biology Open Software Suite

FBS, fetal bovine serum

FWPV, fowlpox virus

G, guanine

GPCR, G-protein-coupled chemokine receptor

HA, hemagglutinin

HeLa cell, cell line derived from human cervical cancer tissue

IEV, intracellular enveloped virus

IFN, interferon

IFN-BP, interferon binding protein

IFNR, interferon receptor

IMV, intracellular mature virus

IRF, interferon response factor

ITR, inverted terminal repeat

IV, immature virus

kb, kilobases

LAP, leukemia-associated protein

LD_{50}, lethal dose 50%

MCP, membrane co-factor protein

MGF, myxoma virus growth factor

MHC, major histocompatibility complex

MOCV, molluscum contagiosum virus

MOI, multiplicity of infection

MOPICE, monkeypox inhibitor of complement enzymes

MPXV, monkeypox virus (-COP-58, strain Copenhagen; -WRAIR-61, strain Walter Reed 267; -SL-V70, strain Sierra Leone; -ZAI, strain Zaire)

mRNA, messenger ribonucleic acid

MVA, modified vaccinia virus strain Ankara

MYXV, myxoma virus

NAP, Nucleotide-Amino Acid Alignment Program

NK, natural killer

NPH II, nucleoside triphosphate phosphohydrolase II

OAS, 2', 5'-oligoadenylate synthetase

OPV, orthopoxvirus

ORFV, Orf virus

ORF, open reading frame

PCR, polymerase chain reaction

PFU, plaque forming unit

PKR, dsRNA-activated protein kinase

POCs, Poxvirus Orthologous Clusters database

RFLP, restriction fragment length polymorphism

RPXV, rabbitpox virus (-UTR, strain Utrecht; -RI, isolate from the outbreak in Rockefeller Institute)

RT, room temperature

serpins, serine protease inhibitors

SFV, Shope fibroma virus

SWPV, swinepox virus

SPICE, smallpox inhibitor of complement enzymes

SPI, serine protease inhibitor

STAT, signal transducer and activator of transcription

T, thymidine

TNF, tumor necrosis factor

TNFR, tumor necrosis factor receptor

U.S.A., United States of America

VACV, vaccinia virus (-TIA, strain Tiantan; -COP, strain Copenhagen; -MVA, modified vaccinia Ankara; -WR, strain Western Reserve)

VARV, variola virus (- BSH. Bangladesh-1975; -IND, strain India-1967; -GAR, Garcia-1966)

VCP, vaccinia virus complement control protein

VEGF, vascular endothelial growth factor

VEGFR, vascular endothelial growth factor receptor

VGF, vaccinia virus growth factor

VGO, Viral Genome Organizer software

vTNFR, viral tumor necrosis factor receptor

WHO, World Health Organization

WNV, West Nile Virus

YLDV, yaba-like disease virus

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Chapter 1 Introduction

1.1 General Features of Poxviruses

Poxviruses are among the largest of all animal viruses and can be seen using light microscopy. The poxvirus virion contains a noninfectious, linear, dsDNA genome (Moss, 2001). The genome length varies from 134,431 bp for Bovine papular stomatitis virus (BPSV-AR02) (Delhon et al., 2004) to 359,853 bp for Canarypox virus (CNPV) (Lee, Essani, and Smith, 2001) among the 52 completely sequenced poxviruses (http://www.biovirus.org/). There are long inverted terminal repeat (ITR) sequences at each end of the poxvirus genome (Garon, Barbosa, and Moss, 1978) and within these, there are short, tandem direct repeats (DR) (Wittek and Moss, 1980). The lengths of ITRs and DRs vary among the virus species.

The poxvirus genome encodes more than 100 proteins (Essani and Dales, 1979; Oie and Ichihashi, 1981; Sarov and Joklik, 1972). Generally, the genome can be subdivided into three regions: the highly conserved central region and the two more variable terminal regions (Mackett and Archard, 1979). The genes located in the central conserved region tend to be those that are essential for virus replication, mRNA transcription and virion assembly. In contrast, the genes located in the terminal regions tend to be involved in determining host range, or for subverting host defense mechanisms *in vivo* (Moss and Shisler, 2001).

Compared to other animal viruses, poxviruses are unique because they replicate in the host cytoplasm; they encode all the components required for transcription of the three temporal classes of genes (Moss, 2001).

1.2 Poxvirus Life Cycle

The first step of virus infection is the attachment to the host cell, this is followed by penetration of the cell membrane and release of the viral core into the host cytoplasm. The intracellular mature virion (IMV) and extracellular enveloped virion (EEV) have different surface proteins and enter cells by different mechanisms (Carter GC, 2005, May). IMV enters cells by fusion with the host plasma membrane (Chang and Metz, 1976; Janeczko, Rodriguez, and Esteban, 1987). In contrast, EEV enter cells by endocytosis (Ichihashi, 1996; Vanderplasschen, Hollinshead, and Smith, 1998).

After the virus enters the host cell and completes primary uncoating, the early genes are transcribed. The transcription of an early gene is regulated by an A+T rich promoter sequence that is located immediately upstream of the RNA start site and close to the translation initiation codon (Davison and Moss, 1989a). The promoter sequence can be recognized by RNA polymerase (Moss, 2001). A consensus sequence of early promoter (AAAAAATGAAAAAA/TA) has been defined by mutagenesis analysis. In addition, an early gene transcription termination signal sequence (TTTTTNT) is found approximately 20-50 bp downstream of the gene (Yuen, Davison, and Moss, 1987). Many of the early mRNAs are translated into proteins that are needed for transcription of intermediate

genes and genome replication. However, a significant fraction of the early proteins have non-essential functions (Moss, 2001a). In VACV, early mRNAs are detected 20 minutes after infection and reach their peak after approximately 1 to 2 hours after infection (Baldick and Moss, 1993). About half of the VACV genes are early genes (Boone and Moss, 1978; Paoletti and Grady, 1977). Once these early genes are transcribed and translated, the virus undergoes a second round of uncoating which releases the viral nucleoprotein into the cytoplasm to begin viral DNA replication.

DNA replication occurs in the virosomes, a localized region in the host cell cytoplasm (Pennington and Follett, 1974; Prescott, Kates, and Kirkpatrick, 1971). The start time for viral DNA replication varies according to poxviruses species, host cell type and multiplicity of infection (MOI). In VACV, replication starts about 1 hour after infection and up to 10,000 copies of the genome may be produced per infected cell. Although it is not known if VACV has a specific origin for DNA replication (Moss, 2001), transfection of a linear DNA molecule containing VACV hairpin ends indicates that a 200 bp sequence from the VACV telomeres is necessary for optimal template replication (Du and Traktman, 1996) .

Viral DNA replication is followed by transcription of intermediate genes. Generally, the tetra-nucleotide TAAA serves as a core element of intermediate promoters (Baldick, Keck, and Moss, 1992). Intermediate mRNAs begin to appear approximately 100 min after infection and quickly reach their peak, then decline in quantity (Baldick and Moss, 1993; Moss and Salzman, 1968; Pennington, 1974; Vos and Stunnenberg, 1988). The

intermediate mRNAs are translated into late transcription factors (Keck, Baldick, and Moss, 1990), RNA helicase NPH II (Shuman, 1992), a single DNA binding-protein that interacts with ribonucleotide reductase (Davis and Mathews, 1993; Tseng et al., 1999), SPI3 (a member of the serine protease inhibitor super family) (Turner et al., 2000) and several other proteins (Moss, 2001).

The transcription of late genes follows that of intermediate genes. TAAAT is the core element of late promoters with G or A usually following this sequence (Davison and Moss, 1989b). In HeLa cells, late mRNA is detected at 140 minutes after synchronous infection with VACV and may continue for about 48 hours (Moss, 2001). Late genes encode the major virion components and the factors that are specifically required for transcription of early genes (Moss, 2001). The late genes tend to be in the central region of the genome.

Virus assembly starts with the budding of viral crescents in circumscribed, granular, electrondense areas of the cytoplasm. The crescent consists of a single bilayer membrane that is not continuous with cellular organelles (Moss, 2001; Sodeik and Krijnse-Locker, 2002). However, how such a membrane could form *de novo* is unclear. The crescents mature further into spherical, immature viruses (IV), which are filled with a similar granular matrix. Then, the DNA is packaged into IV. The IVs containing viral DNA mature into IMV in a process that requires the proteolytical cleavage of A3L, A10L and L4R proteins (Jensen et al., 1996). Some of the IMVs are wrapped by membranes that are accessible to endocytosed molecules and contain markers of the trans-Golgi network, and

form IEVs. The IMVs and IEVs are transported by the cytoskeleton through the cytoplasm and upon reaching the plasma membrane, IEVs fuse with this membrane releasing EEV into the extracellular medium (Moss, 2001).

1.3 Classifications of Poxviruses

The Poxviridae was classified into two subfamilies based on the host range (Moss, 2001): the Chordopoxvirinae (ChPV) infect vertebrates whereas the Entomopoxvirinae infect invertebrates and insects. There are eight genera: Orthopoxvirus (OPV), Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus, and Yatapoxvirus. Entomopoxvirinae consist of three genera: Entomopoxvirus A, Entomopoxvirus B and Entomopoxvirus C. Members of the same genus are genetically and antigenically related and have a similar morphology (Moss, 2001). The OPV genus of the ChPV family includes the most infamous variola virus (VARV) that once caused the devastating disease of smallpox, and the prototypic member, VACV, that is used as the smallpox vaccine.

1.4 Poxvirus Pathogenesis

Different poxviruses use a variety of routes to facilitate their transmission to their target organisms. For example: molluscum contagiosum (MOCV) infects humans through the skin; VARV infects humans via the respiratory tract (Fenner, 1988); ectromelia virus (ECTV) infects mice primarily through minor abrasions of the skin (Fenner, 1948a);

myxoma virus (MYXV) and Shope fibroma virus (SFV) are spread by insect vectors among their rabbit hosts (Cameron et al., 1999; Willer, McFadden, and Evans, 1999); swinepox virus (SWPV) primarily uses lice as a vector (Afonso et al., 2002); zoonotic MPXV infections probably occurs through small lesions on the skin, the oral mucous membrane, or by the respiratory route in cases of person to person transmission (Moss, 2001).

Most poxvirus infections cause acute infections with localized lesions or systemic infections depending on the virus species. In experimental monkey infections, VARV and MPXV cause a systemic illness with fever and rash, similar to that in humans (Moss, 2001); cowpox virus (CPXV) and VACV usually cause a localized dermal infection; ECTV causes a systemic infection of mice under experimental conditions. In contrast, MOCV replicates only in the *stratum spinosum* of the human epidermis without involvement of the dermis; it does not spread systemically from the site of infection, although it may be spread by mechanical re-infection (Moss, 2001).

Poxviruses encode proteins involved in blocking many of the strategies employed by the host to combat viral infections (Moss, 2001). These genes have been classified into three groups according to their functions (Nash et al., 1999): i) virostealth proteins mask the signals associated with virus infection, ii) virotransductor proteins stop host cell intracellular signals such as those required for apoptosis, and iii) viroceptor/virokine proteins block extracellular communication.

1.4.1 Virostealth

Virostealth is characterized by concealing the presence of infected cells from the immune system.

The innate and cytolytic immune cells, such as NK cells and CTLs, are critical for the rapid identification and clearance of virus-infected cells (Barry and Bleackley, 2002). MHC I molecules play a key role in these processes, because MHC I present endogenous viral antigens to circulating $CD8^+$ CTLs. In addition, NK cells can distinguish a reduction in the display of class I MHC molecules on the surface of virus-infected cells, and kill them directly (Richard et al., 2003). Studies show that the MYXV 153R protein disrupts MHC I antigen presentation. MYXV 153R is an early gene and its product contains a leukemia-associated protein (LAP) domain, it targets MHC I molecules both at the cell surface and in the post-Golgi compartment for retention and degradation in lysosomes (Guerin et al., 2001). Moreover, the LAP domain of MYXV 153R also mediates MYXV-induced CD4 downregulation, the LAP domain is thought to be involved in the ubiquitination of CD4 and to promote the lysosomal internalization and degradation of the receptor (Mansouri et al., 2003). An ortholog of MYXV 153R is also present in the genomes of SFV, SWPV, and Yaba-like disease virus (YLDV).

Another mechanism to interfere with the MHC I pathway involves the MOCV 080R gene which encodes an MHC I homolog. It is believed that this may act as a ligand for an inhibitory NK cell receptor (Senkevich and Moss, 1998). SWPV (Afonso et al., 2002)

and YLDV (Lee, Essani, and Smith, 2001) also contain genes that encode proteins with similarity to MHC I domains. The above studies indicate that down-regulation of the host MHC I system may be a common virostealth mechanism among poxviruses.

1.4.2 Virotransduction

The virotransduction strategy, which is carried out by intracellular viral proteins, seeks to retard innate antiviral responses such as apoptosis, and block the communication between the infected cell and the cellular arm of the immune system (Nash et al., 1999). Since blocking the generation of the progeny virions and their dissemination is an important host defense, it is not surprising that poxviruses have evolved strategies to moderate these processes in infected cells. Among the viral strategies that block host cell signal transduction, those that inhibit the apoptosis pathways, are particularly important (Barry, 2004).

After infection, poxviruses rapidly express proteins that inhibit the host cell apoptotic cascade. These inhibitory viral proteins can be divided into two classes: those that block caspase activation, and those that serve as suicide caspase substrates. Shisler and Moss confirmed that MOCV 159L and 160L inhibit host cell apoptosis (Shisler and Moss, 2001), but the mechanism by which these two genes function is not clear. Some studies show that both MOCV 159L and 160L contain two death-effector domains (DED) which can bind both caspase-8 and the Fas-associated death domain adaptor, and therefore prevent the apoptosis induced by death receptors (Thome et al., 1997). Others suggested

that MOCV 159L exerts its inhibitory effects by interacting with cellular factors other than its predicted binding partners within the death receptor complex because the DEDs of MOCV 159L cannot be functionally interchanged with those from other host proteins (Garvey et al., 2002a) and mutations within regions of MOCV 159L other than those within the DED motifs eliminate its anti-apoptotic activity (Garvey et al., 2002b).

Other apoptosis inhibitors include some serine protease inhibitors (serpins), which are a group of proteins that regulate complex proteinase-dependent pathways involved in the apoptosis, inflammation, and tissue remodeling (Shi et al., 1992). Here, we focus on their anti-apoptotic function in poxvirus infections. The CPXV serpin cytokine response modifier A (CrmA) inhibits apoptosis through both endogenous and exogenous apoptotic pathways (Ray et al., 1992). CrmA protects cells from perforin-dependent apoptosis induced by CTLs and NK cells by inhibiting the serine protease granzyme B, a protein that causes the most rapid cell death rate (Quan et al., 1995; Shi et al., 1992). In addition, CrmA has a unique structure (Stittelaar et al., 2000) and has a cross-class caspase inhibitory activity and blocks caspase-8 and -10 by functioning as a caspase suicide substrate; it thereby blocks the apoptotic pathways that are initiated by serum and growth factor deprivation, hypoxia, detachment from the extracellular matrix, or tumor necrosis factor (TNF) and Fas ligation (Born, 2000; Gurevich, 2001; Tewari and Dixit, 1995). The SPI-2 family of poxvirus serpins also has the potential to inhibit apoptosis; VACV SPI-2 protects cells from Fas- and TNF-mediated apoptosis (Dobbelstein and Shenk, 1996). The MYXV serine protease inhibitor MYXV 151R inhibits both caspase-1 and granzyme B to protect infected lymphoid cells from apoptosis (Messud-Petit et al., 1998).

Mitochondria also play a role in the generation of apoptotic signals initiated by cellular stress (Castedo, 2002; Miller et al., 1998; Wang et al., 2001). The MYXV 11L protein interacts with the peripheral benzodiazepine receptor, a component of the mitochondrial permeability transition pore, and thereby inhibits pro-apoptotic changes in mitochondrial integrity such as the loss of inner mitochondrial membrane potential (Everett et al., 2000; Everett et al., 2002; Wisser et al., 2001). Similar protection of the mitochondrial membrane potential has also been observed after infection by VACV (Barry and Bleackley, 2002; Everett et al., 2002; Ramsey-Ewing and Moss, 1998), but VACV does not have an obvious ortholog of MYXV 11L, therefore the gene responsible in VACV is still not known.

Poxvirus anti-apoptotic activities also target the interferon (IFN) inducible enzymes protein kinase R (PKR) and 2′, 5′-oligoadenylate synthetase (OAS) (Seet et al., 2001). Both of these enzymes are activated by dsRNA which is produced during viral transcription and initiate cascades that inhibit viral protein synthesis or induce apoptosis by activating caspase-8 (Gil, Esteban, and Roth, 2000). VACV E3L and K3L genes express the prototypical poxvirus inhibitors that target these enzymes; VACV E3L is a dsRNA-binding protein that sequesters dsRNA and prevents activation of PKR and OAS and also blocks apoptosis (Chang, Watson, and Jacobs, 1992; Davies et al., 1993). E3L can also bind directly to PKR to inhibit its activity, thereby preventing the phosphorylation of eukaryotic initiation factor 2α (eIF-2α) and the IFN response factors 3 and 7 (IRF-3 and -7) that are associated with cell cycle arrest (Sharp et al., 1998; Smith et

al., 2001). In comparison, the K3L gene product, a structural mimic of the eIF-2α subunit, functions as a suicide pseudosubstrate of PKR to competitively inhibit eIF-2α phosphorylation (Carroll et al., 1993; Davies et al., 1993). Orthologs of E3L and/or K3L are also detected in the genomes of MYXV, YLDV, VARV, SFV, ECTV, ORFV, and SWPV.

Finally, poxviruses can also interfere with apoptosis through elements within IFN pathways other than PKR and OAS. For example, the VACV H1L gene encodes a dual-specificity phosphatase that blocks IFN-induced activation of the signal transducer and activator of transcription 1 (STAT-1) (Najarro, Traktman, and Lewis, 2001) and MOCV uses 159L to inhibit IFN-mediated, PKR-induced NF-κB activation (Gil et al., 2001).

To summarize, the poxviruses encode numerous different proteins to subvert the host apoptosis process and this mechanism plays an important role in poxvirus pathogenesis.

1.4.3 Viromimicry: Viroreceptors and Virokines

This group of poxvirus proteins subverts the host immune defense by mimicking the function of host immune molecules. Generally, these proteins target the host's extracellular pathways, which are related to regulation of early inflammatory responses, particularly at the level of complement, IFNs, proinflammatory cytokines, cytokines, and growth factors. According to their activity, these proteins have been classified into two groups: viroreceptors and virokines. Viroreceptors, which may be either secreted or localized to the surfaces of infected cells, are related to cellular receptors and act by scavenging ligands that promote antiviral immune or inflammatory processes. In contrast, virokines are secreted viral proteins that mimic host immune molecules.

IFN viroceptors that target IFN-α/β and IFN- γ are critical for host anti-viral responses; all poxviruses employ extracellular and/or intracellular mechanisms to disrupt IFN activity (Sen, 2001). Viral mimics of IFN-receptors represent the most common poxvirus extracellular strategies to evade the antiviral effects of these cytokines (Smith, Symons, and Alcamí, 1998). Poxvirus IFN binding proteins (IFN-BP) are similar to the extracellular domains of mammalian IFN-α/β and/or IFN-γ receptors, and therefore competitively prevent host IFNs from binding to their native receptors (IFN-Rs) (Smith, Symons, and Alcamí, 1998). The VACV B8R encodes a protein that binds IFN- γ from several species (Alcami and Smith, 1995). The protein encoded by VACV B18R is secreted and localizes to the surface of infected and uninfected cells and therefore protect infected cells from the direct action of IFN-α/β and uninfected cells from IFN-induced

resistant to infection (Alcami, Symons, and Smith, 2000). In addition, MYXV T7 is a homolog of IFN- γ receptor (Upton, Mossman, and McFadden, 1992), its anti-IFN properties are rabbit-specific (Mossman et al., 1996a).

Poxviruses also target the IFN pathway indirectly through proteins that scavenge IL-18, a pro-inflammatory cytokine that induces IFN-γ production. Many poxviruses encode soluble homologs of mammalian IL-18 binding protein (IL-18 BP) (Smith, Bryant, and Alcami, 2000; Xiang and Moss, 1999). The IL-18 BPs encoded by VACV, ECTV and CPXV, exhibit much greater affinity for murine IL-18 than the human IL-18 (Calderara, Xiang, and Moss, 2001). This suggests that the natural hosts for these viruses are more closely related to mice than humans. Human poxvirus MOCV 51L, 53L and 54L also encode three putative IL-18 BPs homologs, but only MOCV 54L appears to bind to human and murine IL-18 with high affinity (Xiang and Moss, 1999).

TNF is a potent proinflammatory cytokine secreted by macrophages and activated T cells. To inhibit the activity of this cytokine, many poxviruses encode soluble proteins that resemble secreted versions of the extracellular domains of the cellular TNF receptor (TNFR), termed vTNFRs (Cunnion, 1999). vTNFRs function primarily as molecular scavengers that bind to and sequester TNF (Barry and McFadden, 1997; Xu, Nash, and McFadden, 2000). The secreted glycoproteins T2-like vTNFRs found in MYXV and SFV bind TNF with high affinity (Sedger and McFadden, 1996). SFV T2 has been reported to bind both TNF- α and TNF-β in several species (Smith et al., 1992), but MYXV T2 exhibits specificity for rabbit TNF- α (Schreiber, Rajarathnam, and McFadden, 1996).

Other well characterized vTNFRs include CrmB, CrmC, CrmD, CrmE and a putative new fifth member from CPXV (Saraiva and Alcami, 2001; Cunnion, 1999; Panus et al., 2002). CrmD is found primarily in poxviruses that lack CrmB and CrmC (Alcami et al., 1999; Cunnion, 1999). Functional CrmE has been identified only in CPXV and VACV USSR strain; it only inhibits human TNF-mediated cytolysis although binds TNF from several hosts species (Reading, Khanna, and Smith, 2002; Saraiva and Alcami, 2001).

Many poxviruses also encode proteins that resemble mammalian growth factors. These impact various aspects of virus virulence and spread, including promoting epithelial growth factor receptor (EGFR) autophosphorylation and the generation of mitotic responses, as well as reducing EGFR downregulation and degradation to prolong the duration of proliferative signals (McFadden and Graham, 1994). The most notable among them are the homologs of epidermal growth factor (EGF) (Seet et al., 2003a). The EGF homologs encoded by VACV and MYXV, termed VGF and MGF, respectively, are secreted proteins produced early in infection that compete with cellular EGF for receptors expressed on epithelial cells overlying sites of infection (Opgenorth et al., 1992; Stroobant et al., 1985).

The Parapoxviruses ORFV, Bovine Papular Stomatitis Virus (BPSV) (Delhon et al., 2004) and pseudocowpoxvirus (PCPV) (Seet et al., 2003b) encode a homolog of mammalian vascular endothelial growth factor (VEGF) that has been shown to stimulate proliferation of vascular endothelial cells and promote vascular permeability (Wise et al., 1999). The homologs of VEGF encoded by these three parapoxviruses are unique

because their receptor binding is specific to VEGFR-2 and neuropilin-1 (Meyer et al., 1999), suggesting that their function in pathogenesis is likely to contribute to the proliferative and highly vascularized nature of the virus lesions.

Semaphorins are a highly conserved family of immunological regulatory molecules found in animals ranging from invertebrates to mammals (Comeau et al., 1998; Gardner et al., 2001; Goshima et al., 2002; Seet et al., 2003a). The defining characteristic of semaphorins is the presence of a SEMA domain, a region containing approximately 500 amino acids within the extracellular component that mediates receptor-binding specificity (McFadden and Graham, 1994; McFadden and Kane, 1994). Semaphorins are involved in both neuronal development and activation of B and T lymphocytes (Hall et al., 1996; Kumanogoh et al., 2005; Shi et al., 2000). The most notable poxvirus semaphorins are the proteins encoded by VACV A39R and ECTV-MOS 139 genes, which have been shown to possess the defining 500 amino acid 'SEMA' domain (Comeau et al., 1998; Gardner et al., 2001). Surprisingly, studies into the function of the A39R homolog encoded by ECTV 139 have suggested that there are pro-inflammatory properties which manifest as increased recruitment of immune cells to sites of infection because of the upregulation of IL-6 and IL-8 (Comeau et al., 1998). This strategy likely favors virus dissemination within the host by attracting immune cells that can be subsequently infected.

As mentioned previously, serpins are a family of serine proteases inhibitors that regulate complex proteinase-dependent pathways involved in the processes of inflammation, apoptosis and tissue remodeling. The MYXV SERP-1 was the first confirmed secreted

poxviral serpin (Upton et al., 1990). The SPI-3 protein secreted from CPXV-infected cells shares limited sequence similarity with SERP-1, but the presence of a common P1 Arg residue in the active site of the protein confers a similar inhibitory profile *in vitro* (Turner et al., 2000). Both proteins inhibit a range of trypsin-like serine proteinases *in vitro* (Lomas et al., 1993; Nash et al., 1998; Turner et al., 2000), suggesting that the primary function of these serpins is to modulate host inflammatory responses to infection, but although the sequences of SERP-1 and SPI-3 are similar, their functions are not interchangeable (Wang et al., 2000). Details of the mechanism have not been worked out, but a study has shown that the purified SERP-1 interacts with native vascular urokinase-type plasminogen activator receptors to inhibit inflammatory cell responses in a mouse model (Dai et al., 2003).

Chemokines are small, secreted cytokines that contribute to the host's efforts to limit virus infections by attracting leukocytes that mediate inflammatory responses to the infection areas (Richard et al., 2003). Most poxviruses encode chemokine binding proteins (CBPs) or ligand mimics (Mahalingam et al., 2001). Putative G-protein-coupled chemokine receptor (GPCR) homologs and chemokine ligand mimics have been identified in several poxviruses (Alcami, 2003). The roles of CBPs in poxvirus virulence have been examined extensively. CBPs are classified into Type I (low affinity) and Type II (high affinity) in poxviruses. MYXV T7 is the sole Type I poxvirus CBP. The capacity of MYXV T7 to inhibit the activity of a broad spectrum of chemokines, in addition to IFN-γ, appears to arise from its ability to interact with the heparin binding domains common to many chemokines (Lalani and McFadden, 1997). MYXV T7 has the potential

to interfere with generalized chemokine binding to glycosaminoglycans and disrupts the localization of a large number of chemokines in tissues. Deletion of the MYXV T7 gene prevents dissemination of the virus to distal sites of infection (Mossman et al., 1996b). In addition, loss of MYXV T7 function is associated with leukocyte infiltration into the primary dermal sites of viral replication and activation of leukocytes in secondary immune tissues, such as the lymph nodes and spleen (Mossman et al., 1996b). Type II CPB, a 35kDa protein encoded by many OPVs (Smith et al., 1997) and MYXV T7 (Graham et al., 1997), target the normal GPCR ligands and competitively inhibit their activities (Seet and McFadden, 2002). The *in vitro* studies indicated that Type II CPB proteins bind chemokines and impede leukocyte migration. Surprisingly, deletion of type II CBP appears to have limited effect on the virulence *in vivo* (Lalani et al., 1999). Similarly, an infection with RPXV lacking the CBP-II gene also differs little from wild-type RPXV infection in mice (Martinez-Pomares, Thompson, and Moyer, 1995).

The complement system is an integrated network of cell-associated effecter proteins and secreted regulatory proteins that participate in the identification and destruction of invading pathogens, as well as the initiation and amplification of inflammatory responses (Richard et al., 2003). The VACV complement control protein (VCP) has been well studied and represents the prototypic poxviral strategy to modulate this system. VCP targets both the classical and alternative complement activation pathways by directly and indirectly promoting the decay of the C3 convertase that converts inactive C3 to active C3a and C3b (Kotwal et al., 1990; Richard et al., 2003; Sahu et al., 1998). The homolog of VCP is also present in several other OPVs (Kotwal, 2000). Interestingly, smallpox

inhibitor of complement enzymes (SPICE) functions similarly to VCP but SPICE inhibits human C3 activity nearly 100-fold more efficiently than VCP (Rosengard et al., 2002).

1.5 Current Status of Poxvirus Research

Smallpox that was caused by VARV, one prototype of poxvirus, was finally eradicated in 1977; this was achieved by using prophylactic inoculations with VACV for many years through a dedicated effort spearheaded by the World Health Organization (WHO). However, new problems with poxvirus infections have emerged. Current research on poxviruses mainly targets these new problems. First of all, since viruses are constantly undergoing an evolutionary process through natural selection, it is possible that someday a relatively non-virulent or less virulent virus in humans, such as MPXV, might develop into a highly virulent smallpox-like virus, or that a highly virulent virus of non-primates, such as RPXV, could jump the species barrier and infect humans. Therefore characterization of the virulence mechanisms of MPXV and RPXV are important areas of research, especially after the MPXV outbreak in U.S.A. in 2003. A second major area of current poxvirus research involves the development of a new smallpox vaccine with fewer side effects. Since vaccination for smallpox was stopped in the1980s, much of the world's population is now susceptible to infections of smallpox, MPXV, and other poxviruses. Natural infections, or those caused by terrorist activities, could result in a global pandemic. Moreover, the side effects of the current smallpox vaccine remain a significant concern (Lane and Millar, 1969; Maurer, Harrington, and Lane, 2003). Thirdly, our understanding of the mechanisms by which poxviruses modulate key

components of the immune system has contributed to the exploitation of viral immunomodulatory proteins for therapeutic use. For example, several poxvirus proteins, including MYXV SERP-1 (Miller et al., 2000), MYXV T7 (Liu et al., 2000), MOCV 148R (DeBruyne et al., 2000), and VCP (Anderson, Smith, and Kotwal, 2002), were used in animal models to prevent allograft and xenograft transplant rejection. MYXV SERP-1 and MYXV T7 were also used to inhibit adverse inflammatory responses in models of arterial injury following balloon angioplasty (Liu et al., 2000; Lucas et al., 1996). In addition, the anti-inflammatory properties of CBP-II encoded by CPXV are promising in inhibiting bronchospasm and cellular infiltration in models of asthma (Dabbagh et al., 2000). Finally, some less virulent poxviruses are being used as live-vector vaccine candidates because they are capable of accommodating large numbers of foreign genes, and could possibly promote expression of a large number of foreign proteins, thereby eliciting a broad immune response. For example, CNPV (Adler et al., 1999; Karaca et al., 2005), modified vaccinia Ankara (MVA) (Seth et al., 1998) and FWPV (Skinner et al., 2005) have been used as live-vector virus vaccine candidates for diseases such as AIDS and West Nile virus (WNV) (Chen et al., 2005; Gorse et al., 2001; Minke et al., 2004).

In addition, as a new and promising field, bioinformatics analysis is able to provide new insights in many areas of poxvirus research. The Poxvirus Bioinformatics Centre (http://www.poxvirus.org; http://www.biovirus.org) provides a variety of useful tools for the rapid identification, annotation, comparison and characterization of poxvirus genomes (Lefkowitz et al., 2005; Upton, 2004). For example, VOCs is a potent tool for the analysis and alignments of poxvirus gene families (Ehlers et al., 2002); VGO is a java

based interface for viewing and searching genomes, and allows the user to identify related genes in multiple sequences (Upton et al., 2000); JDOTTER is a interactive Dot-Plots of complete poxvirus genomes (Brodie, Roper, and Upton, 2004); BBB is a powerful and convenient software for genome alignment analysis (Brodie et al., 2004).

1.6 Introduction of Rabbitpox Virus

RPXV is a member of the chordopoxvirus subfamily and the OPV genus of poxviruses. In 1932, Greene, a scientist at the Rockefeller Institute of Medical Research in New York working on VACV, observed in the Institute's large breeding stock of rabbits a highly lethal epidemic disease, involving fever and often a rash. The poxvirus responsible for the disease was designated rabbitpox-Rockefeller Institute (RP-RI) virus (Fenner, 1958; Greene, 1933). In 1941 in Utrecht, the Netherlands, a similar disease occurred and the virus isolated from this outbreak was designated RPXV-UTR (Esposito and Fenner, 2001; Fenner, 1994).

Generally, RPXV was thought to be derived from VACV because it arose in laboratories where work on VACV in rabbits was ongoing, and its genome is similar to VACV by restriction endonuclease analysis (Wittek et al., 1977). In addition, many of the clinical, pathological and virological features of RPXV are similar to those of VACV (Greene, 1933; Greene, 1933a; Greene, 1934b); however, RPXV is highly lethal in rabbits, while VACV is not. VACV passaged in the skin of rabbits fails to produce a fatal disease and no severe infections are observed with aerosol infection doses as high as 1.3×10^4 plaque

forming unit (PFU). In contrast, similar aerosol infection with RPXV produces almost uniform fatalities with a dose of 15 PFU/rabbit (Westwood et al., 1966). Studies determined that VACV and RPXV replicate to similar titres in the rabbit lung, but only RPXV showed consistent and significant titres in internal organs such as spleen, liver, kidney, gonads and brain, which suggests that dermal-VACV is unable to disseminate from the lung (Westwood et al., 1966). Up to now, the molecular basis of RPXV's enhanced virulence for the rabbit, as compared to VACV, was unknown.

Another reason for studying RPXV is related to its use as a non-primate animal model for smallpox infection. Since RPXV is spread via the respiratory tract, it may be a second good animal model for the study of smallpox in addition to ECTV in mice, with which the early experiments on the pathogenesis of generalized exanthematous poxvirus infections were carried out (Fenner, 1948a; Fenner, 1948b; Fenner, 2000; Schriewer, Buller, and Owens, 2004).

1.7 Introduction of Monkeypox Virus

MPXV is the most important OPV infection in humans since the eradication of smallpox in the 1970s (Fenner, Henderson, and Arita, 1988). This is because it can infect humans and cause smallpox-like illness; there is, however, inefficient human-human transmission (Esposito and Fenner, 2001; Esposito and Fenner, 2001). MPXV was first isolated and reported as a disease of Asian cynomolgus monkeys in 1958 (Von Magnus et al., 1959). In the 1960s, MPXV outbreaks with animal fatality rates of 3% to 48% were reported,

but no infections were reported among humans; this is probably because humans had received a cross-reactive preventative smallpox vaccination. In 1970, it was first reported that MPXV can infect humans and cause a smallpox-like disease in the Basankusu Territory of the Democratic Republic of Congo (Ladnyj, Ziegler, and Kima, 1972). Since then, the illness has also been observed as a rare zoonosis with low inter-human transmission in tropical rain forests in West and Central Africa.

Generally, human MPXV is a rare viral zoonosis, endemic to Central and West Africa (Moss, 2001). However, the West African MPXV is genetically distinct from the Central African MPXV as determined by restriction fragment length polymorphisms (RFLP) and DNA sequencing analysis of the hemagglutinin and TNFR genes (Mukinda et al., 1997; Esposito and Fenner, 2001; Esposito et al., 1988). The virulence and transmissibility differences between the West African and the Central African MPXV are supported by the fact that epidemiological analyses observed a similar prevalence of antibodies against MPXV antigens in non-vaccinated humans in both regions (Jezek and Fenner, 1988), while >90% of reported cases occurred in Congo basin of the Central Africa, and no fatal cases were observed outside of this region (Esposito and Fenner, 2001).

Ecological and serologic studies indicate that MPXV has a broad host range. Humans and primates are accidentally infected; squirrels are among the animals that maintain a reservoir of MPXV in the wild animals while sporadically causing human disease (Gispen, 1975). This precludes global eradication by human vaccination alone.

The case-fatality rate of human MPXV illness in DRC was ~10% compared to 30% for VARV major, the causative agent of smallpox (Jezek, Khodakevich, and Szczeniowski, 1988). Unlike smallpox, however, transmission of MPXV between humans is inefficient; more than two generations of transmission from an index case have rarely been documented (Jezek et al., 1988). Prospective studies suggest disease incidence is increasing due to encroachment of humans into habitats of animal reservoirs for MPXV, such as traveling, hunting for food in area of Africa forest, and handling infected animals (Hutin et al., 2001; Mukinda et al., 1997). This proposition is supported by the MPXV outbreak in the U.S. midwest from April to June of 2003 (Reed et al., 2004). A clear understanding, however, of the virulence and transmissibility of human MPXV has been limited by inconsistencies in epidemiological investigations.

Laboratory diagnosis is important because MPXV can cause disease symptoms that are very difficult to distinguish clinically from other pox-like illnesses, particularly smallpox and chickenpox (a herpes virus). Immunization with VACV, the smallpox vaccine, is roughly 85% effective in preventing human MPXV, but there is currently no proven drug treatment for human MPXV infection (Di Giulio and Eckburg, 2004).

1.8 **Thesis Rationale and Objectives**

There are two sections in my research thesis. The first concerns RPXV, and the second examines MPXV.

As noted previously, RPXV is similar to VACV but highly virulent in rabbits. RPXV has long been suspected to be a close relative of VACV because it arose in laboratories where VACV was passaged in rabbits, and is similar to VACV by restriction endonuclease analysis (Wittek et al., 1977) as well as clinical, and pathological features (Greene, 1933; Greene, 1934a; Greene, 1934b). Thus, RPXV may have been present as a natural variant in the crude VACV stocks that were also used for human vaccines. Therefore, my goal was to determine if RPXV is really a variant of VACV and to determine if there are genes present in the genome of RPXV but absent from VACV that could explain the difference in virulence of these two viruses. This information would be valuable for the design of safer VACV-based vaccines since it would be possible to delete these genes from the vaccine candidate.

The MPXV outbreak in the U.S. midwest from April to June of 2003 (Reed et al., 2004) focused significant public and scientific attention on this virus. MPXV entered the U.S. in a shipment of African rodents from Ghana (West Africa), destined for the pet trade. At a pet distribution centre, prairie dogs became infected and, in turn, were responsible for 72 confirmed or suspected cases of human MPXV. However, unlike in Africa, the U.S. outbreak resulted in no fatalities and there was no documented human-to-human transmission (Reed et al., 2004). Although this less severe epizooty could be due to higher natural resistance of the U.S. population, a healthier patient population lacking background infections, and/or better supportive care for patients there is also a significant possibility that this variability in pathogenicity could be due to genetic differences of the MPXV present in the two regions of Africa.

The goal of this thesis was to test the hypothesis that the difference in virulence of these two MPXV groups is due to the presence of specific virulence genes in the more virulent MPXV isolates.

1.9 Contributors to Work Presented in This Thesis

I would like to convey my thanks to the following people for their contribution to the work presented in this thesis.

Dr. R. Mark L. Buller and Dr. Nanhai Chen (Department of Molecular Microbiology and Immunology and Saint Louis University Health Sciences Center) performed the DNA purification, the automated sequencing of RPXV strain Utrecht and MPXV isolates, and the measurement of MPXV inhibitor of complement enzymes activity.

Dr. Elliot J. Lefkowitz, Department of Microbiology, University of Alabama at Birmingham performed part of the phylogeny analysis.

Chapter 2 Material and Method

2.1 Cells and Viruses, Purification of Viral Genome and DNA Sequencing[1]

RPXV-UTR was generously provided by Dr. Dick Moyer of the University of Florida who originally obtained the virus from the American Type Culture Collection (ATCC, catalogue number VR-157). PK 15 cells (CCL-33) were obtained from the ATCC, and propagated in Dulbecco's Modified Eagle's Medium (DMEM, Bio-Whittaker, Walkersville, MD) containing 10 % FETALCLONE II, fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan UT 84321).

MPXV-SL-V70 was obtained from the crusts of lesions from a single human case in Sierra Leone in 1970 (Lourie et al., 1972). The MPXV-WRAIR-7-61 isolate was deposited with the American Type Culture Collection (ATCC, catalogue number VR-267) in May of 1962 by Major Stewart J. McConnell (McConnell et al., 1962). MPXV-WRAIR-7-61 was isolated from a female cynomolgus monkey, B-39, that was observed with a poxvirus-like infection, 45 days following whole-body irradiation of 350 rads (Chen et al., 1992).

[1] This work was performed in the laboratory of Dr. Mark Buller, Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center.

The female cynomolgus monkey (B-39) died 12 days after the onset of disease. The passage history of MPXV-WRAIR-61 after isolation is not known. MPXV-ZAI-V79-I-005 (ZAI-V79) was obtained from scab material of a severe case of human monkeypox in Zaire in 1979, and was passaged sequentially once in LLCMK2 cells, twice in BSC-40 cells, and two or three times in Vero Cells. MPXV-COP-58 was isolated in 1958 from scrapings of several papules on an infected cynomolgus monkey from an outbreak of a vesicular eruptive disease in a primate holding facility (Von Magnus et al., 1959) (see the MPXV used in this thesis in Table 1). The virus was passaged an unknown number of times on the chorioallantoic membrane of the chick egg and in FL, LLCMK2 and BSC-40 cells. Vero, BSC-1 and BSC-40 cells were grown in EMEM (Bio-Whittaker, Walkersville, MD) containing 10 % fetal bovine sera (Hyclone, Logan, UT), 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml of penicillin (GIBCO, Grand Island, NY) and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY).

Table 1 MPXV used *in vivo* virulence studies

West African Isolates	**Central Africa (Congo Basin) Isolates**	**Type of Analysis**
MPXV-COP-58 from monkey in 1958	MPXV-ZAI-V79	Virulence Test in Monkey
MPXV-SL-V70 from human patient in 1970	MPXV-ZAI-96	Genomic Comparison between the West Africa and the Central Africa isolates
MPXV-WRAIR-61 from monkey in 1962	-	-

Five T150 flasks of confluent PK 15 cells (~ 2 x 10^7 cells per flask) were infected with RPXV-UTR or MPXV at a M.O.I. of 10 in 5ml of DMEM, containing 2 % FETALCLONE II. After 1 hr at 37^0 C, cultures were supplemented with a further 20 ml of DMEM-2, and incubated until maximum cytopathic effect was observed (24 hours). Cells were scraped into the culture supernatant and centrifuged at 350 x g for 5 min. The cell pellets were resuspended in a total of 2.4 ml of phosphate buffered saline and divided equally among four 1.5 ml microfuge tubes. The cell suspensions was frozen and thawed three times, mixed with 600 µl of 2 x Triton X 100 buffer (1 % Triton X100, 40 mM EDTA, and 68.5 mM ß-mercaptoethanol), and centrifuged at 850 x g for 5 min. The supernatants were transferred to new microfuge tubes and centrifuged at 21,000 x g for 15 min. The pellet was resuspended in 100 µl of SDS buffer (10 mM Tris, pH 8.0, 200 mM NaCl, 1mM EDTA, 1% SDS, 150 µg/ml proteinase K) and incubated at 50^o C for 30 min. The viral DNA was extracted twice with phenol-chloroform, precipitated by ethanol and resuspended in 50 µl ddH_2O.

With the exception of the terminal hairpin loops, the entire RPXV or MPXV genomes were divided into l8 overlapping fragments of an average size of 11 kb. Each fragment was amplified from genomic DNA using Expand Long Template PCR System (Roche Diagnostics Corporation, Indianapolis, IN) following the manufacturer's instructions. PCR products were purified using either ExoSAP-IT (USB Corporation, Cleveland, OH) or QIAquick PCR Purification Kit (QIAGEN Inc. Valencia, California) 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 either QIAEX II Gel Extraction Kit

(QIAGEN Inc., Valencia, California) if the band was more than 10 kb, or QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, California) if the band was less than 10 kb. Each fragment was sequenced with a bank of sequencing primers which were designed according to the sequence alignment of VARV strain Bangladesh (VARV-BSH) and VACV strain Copenhagen (VACV-COP) (for RPXV), or according to the sequence of MPXV-ZAI (for Western African MPXV isolates). Sequencing primers were about 450 bp apart on each strand to ensure adequate overlap of sequencing reads. Both strands of each fragment were sequenced and gaps were closed by primer walking. Sequencing reactions were carried out using CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Inc., Fullerton, California), and run on CEQ 2000XL DNA Analysis System (Beckman Coulter, Inc., Fullerton, California).

The genomes of MPXV-SL-V70, MPXV-WRAIR-61, and MPXV-COP-58 were sequenced as described above, except that sequencing primers for the sequences of the variable left and right-hand terminal regions were based on MPXV-ZAI-96, the most closely related virus, to ensure the best chance of primers being effective in sequencing.

2.2 Assembly of Sequence Data[2]

For RPXV and MPXV-WRAIR-7-61, assembly of the raw sequence data was performed

[2] The assembly of MPXV-COP-58 and MPXV-SL-V70, two viruses out of four that were sequenced in this thesis, were performed in the laboratory of Dr. Mark Buller, Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center

using the Staden software package on a Linux platform (Dear and Staden, 1991). Raw data was processed through Pregap4, including base calling using Phred (Dear and Staden, 1991), performed using the Staden software package on a Linux platform (Staden, Judge, and Bonfield, 2001), estimating base accuracies, converting trace format, initialising experiment files and quality clipping. The prepared files from Pregap4 were input into GAP4 (Staden, Beal, and Bonfield, 2000). A consensus sequence was assembled and edited gaps were closed with new sequence data using primers based on the assembled contigs.

The primers were designed manually according to the position of gaps and information from the program Oligo Calculator (Eugen Buehler, unpublished; http://mbcf.dfci.harvard.edu/docs/oligocalc.html). The final DNA consensus sequences represented averages of 3.7 (RPXV) and 4.8 (MPXV-WRAIR-7-61) fold redundancy with each nucleotide being covered by at least one high-quality sequence read in each direction. For the assembly of MPXV-COP-58 and MPXV-SL-V70[1], the raw sequence data was assembled using ContigExpress in Vector NTI Suite 8 (Invitrogen, Carlsbad, California); the final DNA consensus sequence had 4.8 fold coverage with each nucleotide being covered by at least one high-quality sequence read in each direction.

2.3 Annotation of Genomes

An open reading frame (ORF) was defined as a continuous stretch of DNA that started at an ATG codon and stopped by a termination codon (TGA, TAA or TAG). In our study, we annotated all the ORF that translated into a polypeptide of at least 60 amino acids (aa); spliced genes are not present in poxviruses. ARTEMIS (Mural, 2000), Poxvirus Orthologous Clusters database (POCs) (Ehlers et al., 2002; Upton et al., 2003), BLASTP (Altschul et al., 1997) and GeneStar software (Windows) (Burland, 2000) were used to detect and annotate ORFs. In addition, for some ORFs, BLASTN, TBLASTN and BLASTP searches were carried out at the NCBI website (Altschul et al., 1997).

RPXV and MPXVs genomes were analyzed with a variety of tools in the POCs package (Ehlers et al., 2002) and others from the European Molecular Biology Open Software Suite (EMBOSS) package (Rice, Longden, and Bleasby, 2002); Motif searches were performed using Motif Scan (Sigrist et al., 2002; Falquet et al., 2002) and the Prosite program. Alignments of DNA and protein sequences were created with ClustalW multiple sequence alignment program (version 1.82) on Linux platform using the default parameters (Thompson, Higgins, and Gibson, 1994) and manually adjusted to maximize parsimony using Base-By-Base (BBB) (Brodie et al., 2004). Single nucleotide polymorphisms (SNPs), insertions and deletions were detected using BBB. Repetitive sequences were found using JDotter (Brodie, Roper, and Upton, 2004) and were further

confirmed using GAP4 and BBB programs. The EMBOSS Showorf program (Rice, Longden, and Bleasby, 2000) and Nucleotide-Amino Acid Alignment Program (NAP) (Huang and Zhang, 1996) were used on a Linux platform to compare the nucleotide sequence of the fragmented ORF regions of RPXV or MPXV against the corresponding longest protein sequence encoded by orthologous ChPV genes. Physical maps of the genomes that included the fragmented ORF regions were made with Omnigraffle 3.1.2 version. The Viral Genome Organizer (VGO) software (Upton et al., 2000) was used to analyze the position and arrangement of genes among multiple genomes.

2.4 Phylogeny Analysis[3]

Phylogenetic analysis was carried out using a genomic nucleotide sequence alignment of the conserved central region of all OPV genomic sequences currently available in GenBank. Sequence alignments were generated using a combination of the programs MAVID and Multi-LAGAN (Bray and Pachter, 2004; Brudno et al., 2003) with manual corrections made using BBB.

[3] Part of this work was performed in the laboratory of Dr. Elliot J. Lefkowitz, Department of Microbiology, University of Alabama at Birmingham.

For RPXV, the alignment extended from base 20,352 to base 163,510 of the genome; this alignment starts with ORF 13 and extends just past ORF 159 of RPXV-UTR and corresponds to the VACV-COP genes C7L through A51R (which is fragmented in RPXV-UTR). The final computer-derived alignment was then extensively hand-edited to optimize the alignment. Phylogenetic inferences were generated using both Maximum parsimony and Bayesian inference methods. Maximum parsimony trees were constructed using PAUP* version 4.0b10 (http://paup.csit.fsu.edu/), while MrBayes, version 3.1 (Ronquist and Huelsenbeck, 2003) was used for Bayesian inference methods. Maximum parsimony trees were constructed using the Branch and Bound search method and employed 1,000 replicates for bootstrap resampling analysis. Bayesian inference using Markov chain Monte Carlo methods used a general time reversible (GTR) model of nucleotide substitution (GTR) and allowed for gamma-distributed variation across sites with a proportion of invariable sites. Tree analysis was performed using 100,000 generations with a sampling frequency of 100. Standard deviation of split frequencies converged to 0 following 45,000 generations, resulting in one final tree with a probability of 99%. In addition, the evolutionary analysis was also performed using protein sequences of the 49 genes that are conserved in all completely sequenced ChPVs using PROTDIST, SEQBOOT and CONSENSE in the PHYLIP package (http://cmgm.stanford.edu/phylip/). NJplot (Linux) (Perriere and Gouy, 1996) and Treeview (Windows) (Page, 1996) were used for construction of the trees.

For MPXV, phylogenetic analysis was carried out using the ~138 kb conserved central region of completely sequenced genomes of various OPV species; the left end of the

alignment extends from gene C7L of VACV-COP (nucleotide 18,805) to A51R at the right end (nucleotide 157,688). The evolutionary relationships were solved using the Branch-and-Bound search method with maximum parsimony as the optimality criterion. Bootstrap resampling confidence values on 1,000 replicates were also calculated using Branch-and-Bound with maximum parsimony. Branch lengths are proportional to the number of sequence changes along each branch. All evolutionary relationships were estimated using PAUP* version 4.0b10 (http://paup.csit.fsu.edu/).

2.5 Hemagglutinin assay

BS-C cells were seeded in 6-well dishes, grew to confluence, and incubated with serial dilutions of RPXV. After 1 h, the cells were washed to remove excess virus and replaced DMEM; the cells were incubated for an additional 3 days, and then fixed cells and stained them with 15% ethanol and 1% crystal violet to visualize plaques. The plaques were observed using a microscope to determine if cell fusion had occurred at their edge; this is an activity of poxviral hemagglutinin (Ichihashi and Dales, 1971).

2.6 Monkey Challenge Experiment and Measurement of MPXV Inhibitor of Complement Enzymes Activity[1]

The collaborator at the United States Army Research Institute of Infectious Diseases performed the monkey challenge experiment. Juvenile to adult, 1.6 to 4.7 kg, cynomolgus monkeys (*Macaca fascicularis*) were challenged by small particle aerosol with mass median diameter of 1.2 μm (Zaucha et al., 2001). The husbandry and experimental protocols were in accordance with *Guide for the Care and Use of Laboratory Animals*. The facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

The comparison of single-cycle replication yields of COP-58 and ZAI-V79. Monolayer cultures of BSC-1 cells were infected with COP-58 or ZAI-V79 at approximately 1 PFU/cell. At 1, 4, 12.5, 24, 34.5, and 46 h post-infection, 4 cultures were harvested for each virus. Cells were scrapped into the cultures supernatant, frozen and thawed 3 times, and infectivity was measured by plaque assay on BSC-1 monolayers.

[1] Animal challenge experiment was performed at the Headquarters, United States Army Research Institute of Infectious Diseases. The comparison of single-cycle replication yields of COP-58 and ZAI-V79, and the measurement of MPXV inhibitor of complement enzymes activity were performed in the laboratory of Dr. Mark Buller, Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center

The MPXV inhibitor of complement enzymes (MOPICE) gene was PCR amplified, cloned and expressed using the *Eco* R1 site of plasmid pSG5 (Stratagene, La Jolla, California) with the enterokinase/6x histidine tag (MOPICE-EH) and without (MOPICE) in Chinese hamster ovary (CHO) cells. Tagged and untagged versions of MOPICE had similar activity. MOPICE protein concentrations were estimated in an ELISA assay. 1:5000 rabbit anti-VCP antibody and horseradish peroxidase goat anti-rabbit IgG was used in the western blot assays. To characterize MOPICE binding to human C4b and C3b, the ligands were coated onto microtiter plates (5 μg/ml in PBS), followed by incubations with media or MOPICE. Binding was detected with rabbit anti-VCP antibody (1:5000) as described above. Chemiluminescent cofactor assays were performed in the presence or absence of 10 ng MOPICE-EH, biotinylated human C3b and C4b and human factor I transferred to nitrocellulose, and probed with avidin- horseradish peroxidase. Final signal development used ECL Plus (Sigma, St. Louis, MO).

2.7 Sequence Availability

The genomes of RPXV-UTR, MPXV-COP-58, MPXV-WRAIR-61 and MPXV-SL-V70 were submitted to GenBank using Sequin software and received the following accession numbers AY484669, AY603973, AY741551 and AY753185, respectively.

The genomes used for comparison are: VACV-Copenhagen: M35027; VACV-Tian Tan: AF095689; VACV-MVA: U94848; VARV-Bangladesh-1975: L22579; VARV-India-1967: X69198; VARV-Garcia-1966: Y16780; MPXV-Zaire-96-I-16: AF380138; ECTV-

Moscow: AF012825; CPXV-Brighton Red: AF482758; CMLV-M96: AF438165; fowlpox virus-Challenge: AF198100; Lumpy skin disease virus NI-2490: AF325528; MOCV subtype 1: U60315; MYXV-Lausanne: AF170726; Rabbit fibroma virus-Kasza: A170722; and SWPV-Nebraska: AF410153.

Chapter 3 Results and Discussion

3.1 RPXV Study

The RPXV study is divided into four parts. The first part describes the general features of the genome and the second part discusses the phylogenetic analysis (part of the phylogenetic analysis was done By Dr. Chunlin Wang in Dr. Elliot J. Lefkowitz's lab). In the third part, the virulence genes are analyzed to determine those most likely responsible for the enhanced RPXV virulence over VACV. In the last section, the fragmented regions in RPXV genome are discussed.

3.1.1 Overview of the RPXV-UTR Genome

The genome of RPXV-UTR was assembled into a contiguous sequence of 197,731 bp. This sequence includes all of the predicted protein coding regions, but does not include the hairpin structures located at each end of the genome. The leftmost confirmed nucleotide, corresponding to part of the HindIII C fragment of VACV, was designated as nucleotide 1 as is customary for annotation of poxvirus genomes. Like most of other OPVs, the RPXV-UTR genome is A+T rich (66.5%); however, it is interesting to note that the individual predicted ORFs range from 55.2% A+T (RPXV-UTR 001) to 73.5% A+T (RPXV-Utr 173). The RPXV-UTR sequence can be subdivided into a central region of 177,687 bp flanked by two inverted terminal repeats (ITRs) of 10,022 bp each (Fig. 1).

These ITR structures are common to all poxvirus genomes although actual sequences differ; the ITRs also contain two sequence blocks made up of direct repeats (DR1 and DR2 in the left ITR, and DR3 and DR4 in the right ITR). DR1/4 is 612 bp in length, including 8.5 copies of a 70 bp tandem direct repeat element and repeat DR2/3 is 363 bp in length, consisting of 6.5 copies of a 54 bp tandem direct repeat element; these are essentially identical to the repeat sequence elements found in VACV-COP. One of the best ways to get an initial overview of the similarity between two large DNA sequences is by using a dotplot. Such an analysis (Fig. 2) revealed that the genomic sequence of RPXV-UTR is a subset of the CPXV-BR genome which is thought to be the ancestor of all modern OPVs (Babkin et al., 2003). However, simply because the DNA sequence is present in both viruses at this gross level does not mean that the genes are functional in either or both viruses. Careful annotation of the genomes is necessary for further meaningful comparisons, hopefully it provides clues to direct experiments that finally determine the function of the genes.

All ORFs that could encode proteins of at least 60 aa were annotated as genes in a first pass annotation. Subsequently, 2 smaller ORFs, RPXV-UTR 010 and RPXV-UTR 123, were added to the annotation due to the existence of highly conserved orthologs in other poxvirus genomes (ORFs CPXV-BR 026 and VACV-COP A14.5L); several genes that were predicted in the first pass were re-classified as "fragmented ORFs" because they

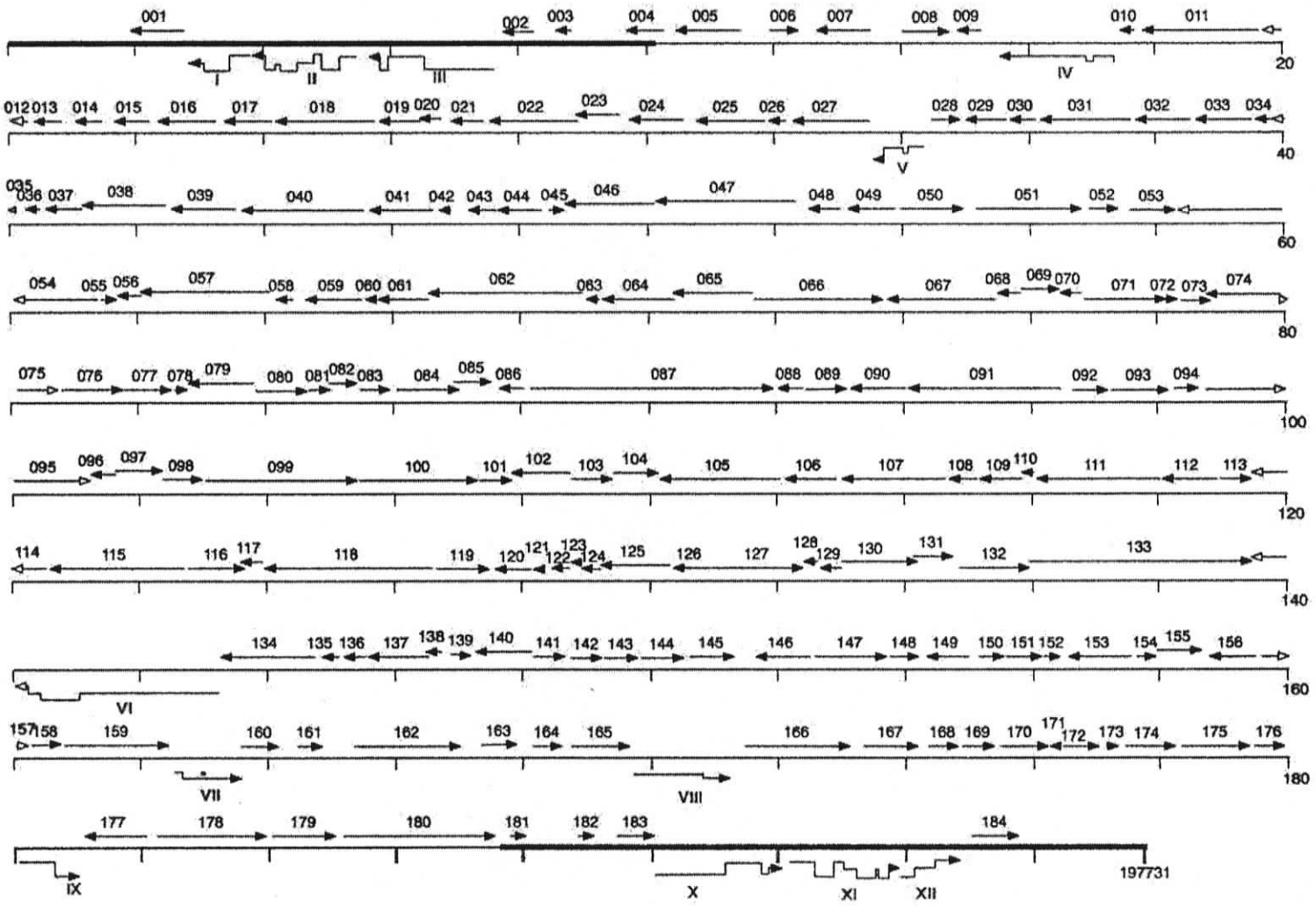


Figure 1 Physical map of the RPXV-UTR genome: predicted genes are numbered and shown as straight arrows; regions containing fragments of genes present in other OPVs are shown with staggered arrows to represent frame changes and have been given roman numerals. Open arrowheads indicate an ORF is split over 2 lines of the diagram; scale is shown in kilobases; thickened lines represent the TIRs of the genome: * above the VII fragment indicates a stop codon.

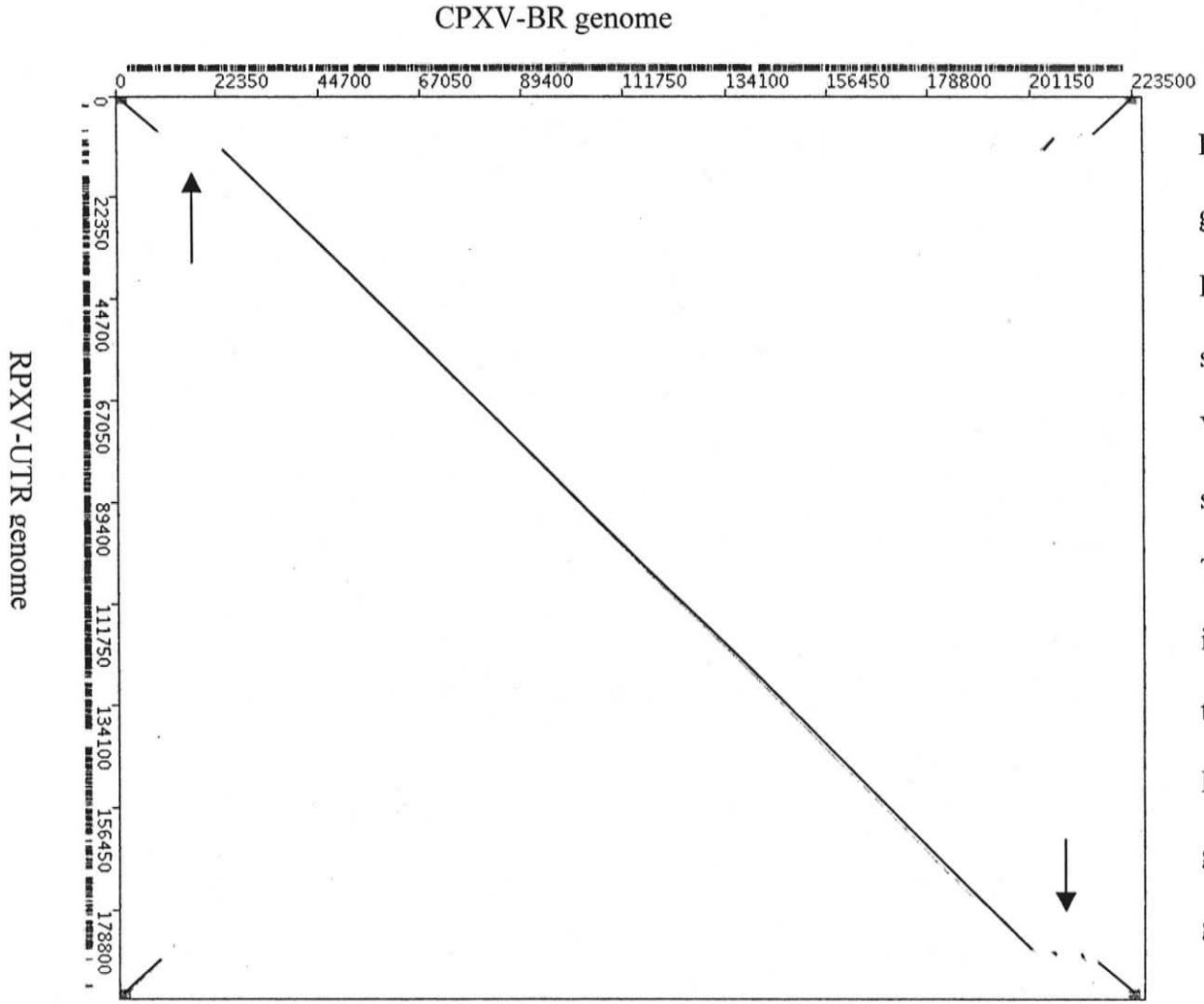


Figure 2 Dotplot of the RPXV-UTR genome and the CPXV-BR genome. Horizontal axis represents the DNA sequence of the CPXV-BR genome. Vertical axis represents the DNA sequence of the RPXV-UTR genome. The arrows show a few big deletions in the RPXV-UTR genome compared to CPXV-BR. Meanwhile, all the DNA sequence in the RPXV-UTR genome is presented in the CPXV-BR genome.

are in fact small regions of significantly larger genes identified in other OPVs and are unlikely to be functional. Thus, in total, we assigned 184 putative genes, predicted to encode proteins ranging in size from 53 to 1,286 aa (Appendix A), and 12 regions that contain fragmented ORFs in RPXV-UTR as compared to other OPVs (Table 3). The 184 putative genes predicted includes 45 genes with unknown function, 45 genes that are related to host range or evasion of host defense, and 94 genes that are thought to be essential for virus replication in standard tissue culture cell lines (e.g. functions mainly necessary for transcribing mRNA, replicating the genomic DNA, and assembling infectious virions) (Table 2). Each ITR contains three complete genes: ORF 001/184, ORF 002/183 and ORF 003/182, as well as, three fragmented ORF regions: I/XII, II/XI, and III/X (Fig. 1). ORF 004 spans the left ITR junction and is an ortholog of CPXV-BR 218; this ORF is fragmented in VACV-COP (annotated as C13L and C14L). Only the C-terminal half of RPXV-UTR 004 is present in the right ITR as RPXV-UTR 181.

Table 2 Summary of the RPXV-UTR annotated ORFs

Function of predicted genes	Number
Essential genes for virus replication in standard cell lines	94
Genes related to host range and the evasion of host defense	45
Genes with unknown function	45

Throughout the RPXV genome, as in other poxviruses, these putative genes and ORF fragments of known genes are located on both strands of the genome with very short sequences between them. The majority of genes present within 50 kb of each terminus are transcribed towards that end of the genome, as is the case with most other poxvirus genomes (Fig. 1). The reason for this arrangement of transcripts is unclear but may be related to minimizing collisions between transcription complexes. Among the 184 predicted genes, 183 genes have annotated orthologs in other OPVs. RPXV-UTR 171, which could encode a 77 aa protein, is the only gene not previously annotated in any fully sequenced poxvirus genome. Despite its significant length, it is probably not a functional gene since there is no promoter-like sequence in the 100 nucleotides upstream of the ORF and the equivalent region in CPXV-BR overlaps the significantly larger CPXV-BR 203 gene that is transcribed in the opposite orientation; CPXV-BR 203 has been experimentally validated as a member of the MYXV T4 virulence factor family (Barry et al., 1997; Hnatiuk et al., 1999).

3.1.2 Phylogenetic Analyses

Phylogenetic relationships among all available OPV genomes were inferred using two different computational methods to ensure that the final evolutionary tree was not dependent on the method used. Inferences based on Maximum parsimony analysis and Bayesian inference each produced a single tree that showed identical topologies and no significant differences in branch lengths (Fig. 3). While confidence values based on bootstrap analysis of the Maximum parsimony tree showed 100% confidence for the

majority of branch points, confidence was lower for a few of the lineages. In contrast, when assessing tree reliability using Bayesian inference, the final tree showed a probability of 99% with the posterior probability of all bipartitions (branch points) equal to 1.0; this provides a great deal of confidence in the final topology.

The DNA tree places RPXV-UTR together with all other VACV strains on one branch, separated from the other OPVs. A similar phylogenetic relationship was obtained for a merged tree created from separate multiple alignments using the predicted protein sequences of the 49 genes that are conserved amongst all completely sequenced OPV (Fig. 4); 25 individual trees grouped RPXV-UTR and four VACV into one clade; other trees did not show significant difference among OPV because the proteins are too similar. These analyses indicate that RPXV-UTR is most closely related to VACV. However, the RPXV-UTR cannot directly be derived from any currently sequenced VACV because we found 719 bps that only present in RPXV-UTR but any currently sequenced VACV (further discussion in the part of virulent gene). How RPXV-UTR compares to the RPXV strain that arose at Rockefeller University is unknown since we have been unable to locate any stocks of the Rockefeller Institute strain of RPXV. The clouded history of VACV together with the fact that it was passaged as a crude mixture in animals at the time that the RPXVs were isolated leaves the connection between these viruses unclear. However, this data supports the hypothesis that RPXV was present as a natural variant in VACV crude stocks and was selected *in vivo* from this complex mixture after infection of rabbits.

According to the phylogenetic tree, VACV is not the most closed relative to VARV. Still, it was successfully used as a smallpox vaccine. This is reasonable not only because both VACV and VARV infect humans efficiently, but also because of the relative lower virulence its host compared to the other strains of OPV (Moss, 2001).

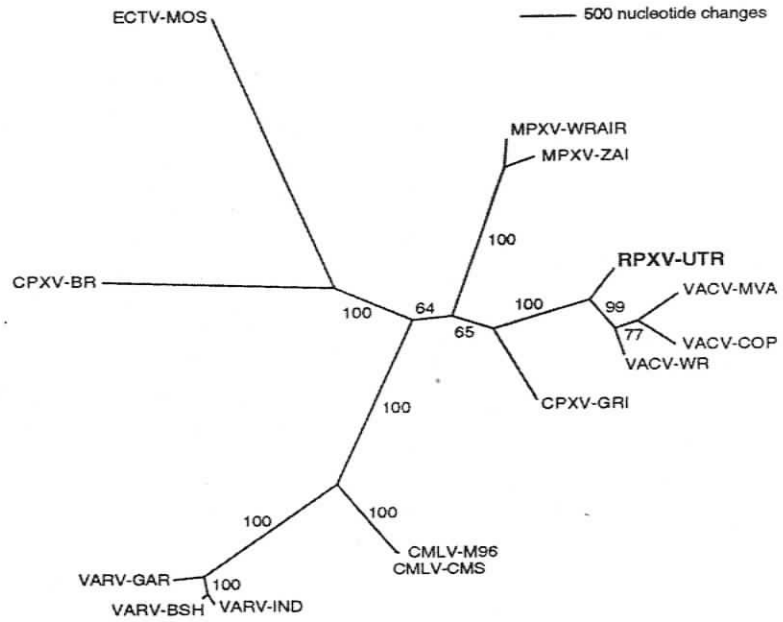


Figure 3 Phylogeny of OPVs. OPV phylogenetic prediction based upon the multiple nucleic acid sequence alignment of the central conserved genomic region of each representative orthopoxvirus species, strain or isolate. The tree represents the phylogenetic inference generated from a Branch-and-Bound search using Maximum parsimony as the optimality criteria. Branch lengths are proportional to the number of nucleotide changes. A tree based on Bayesian inference produced an essentially identical tree. Bootstrap resampling confidence values on 1,000 Branch-and-Bound replicates are displayed for each branch point as a percentage of the total replicates. The posterior probability of all bipartitions (branch points) based on Bayesian inference are equal to 1.0.

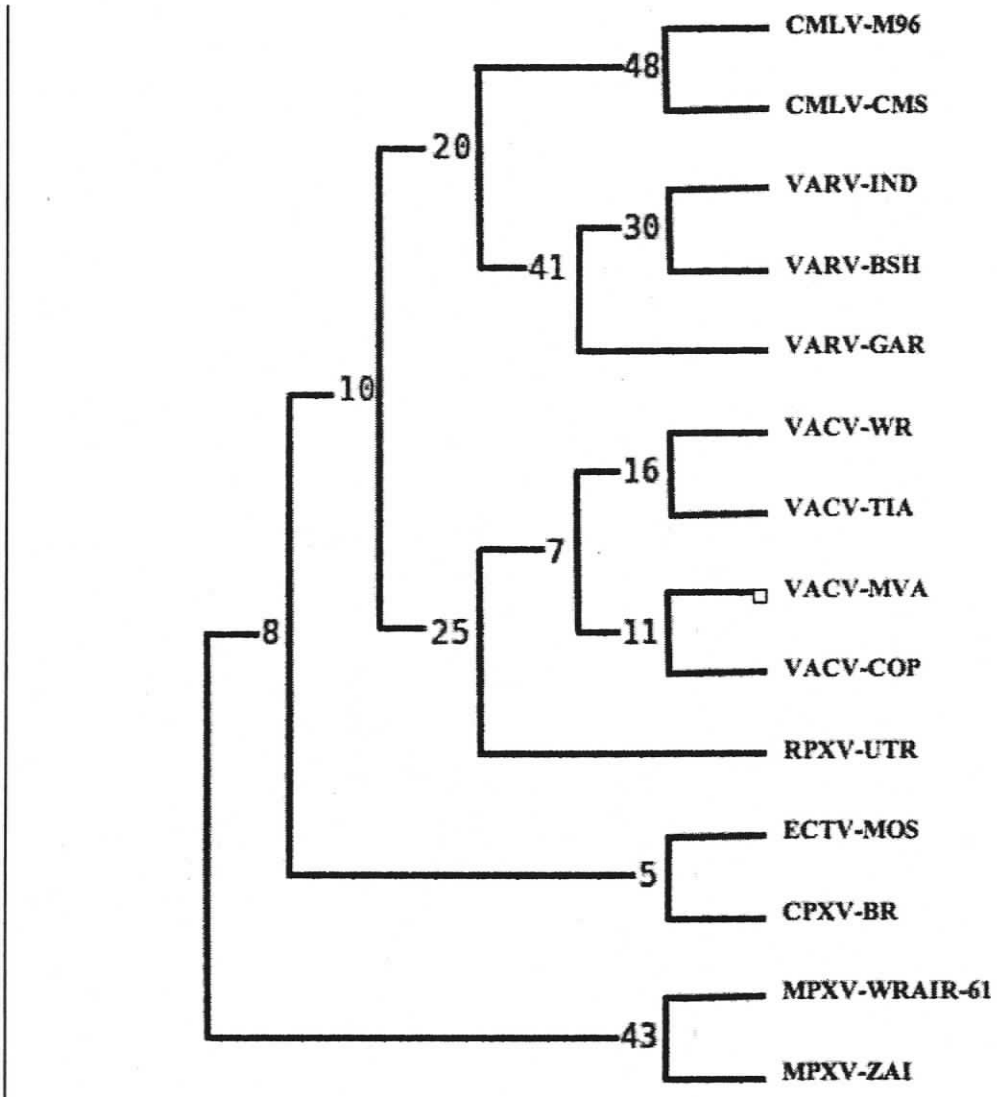


Figure 4 The Merge Tree of the protein sequences encoded by 49 genes conserved among all the completely sequenced OPVs. CLUSTALW was used to align the amino acid sequence of each protein. The 49 protein alignments were used to draw a merge tree using CONSENSE in the PHYLIP Package. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 50 trees.

3.1.3 Virulence Genes

RPXV-UTR was compared with available sequences of strains of VACV and other OPVs to identify ORFs that may contribute to the enhanced virulence of RPXV-UTR over other VACV strains for rabbits. The VACV-MVA sequence was not used in this analysis as it contains a large number of deletions and grows poorly in mammalian cells (Blanchard et al., 1998; Drexler et al., 1998). Furthermore, we have avoided relying heavily on the genome sequence of VACV-TAN because our group, and others, has identified a number of errors in this sequence (Upton et al., 2003). This analysis indicates that RPXV-UTR contains three genes that are not present in VACV-COP or VACV-WR; these genes encode the zinc RING finger protein (ORF 008), an ankyrin repeat containing protein (ORF 180) and a chemokine binding protein (ORF 001/184).

RPXV-UTR 008 is predicted to encode an ortholog of ECTV-MOS 012 gene product (also known as p28), a key ECTV virulence protein (Senkevich, Koonin, and Buller, 1994a). Orthologs are also present in VARV, CPXV, MPXV, CMLV and a number of other ChPVs, but is deleted in VACV-COP and fragmented in VACV-TAN and VACV-WR. The most prominent feature of the predicted ORF 008 protein is a C-terminal zinc binding motif, known as the RING finger motif (PROSITE database PDOC00449); (Sigrist et al., 2002); similar motifs are present in a wide variety of proteins with diverse functions (Boddy et al., 1997; Kaiser et al., 2003; Lovering et al., 1993; Lyngso et al., 2000). This poxvirus protein localizes to the virus factory in the cytoplasm of infected cells (Upton et al., 1994), reduces apoptosis in infected cells (Brick et al., 1998) and has

recently been found to have a ubiquitin ligase activity (Huang et al., 2004; Nerenberg et al., 2005). The predicted protein product of RPXV-UTR 008 is 96.4% identical to ECTV p28, which is not required for virus multiplication in cell culture, but is an important determinant of ECTV pathogenicity. Disruption of this gene in ECTV increases the LD_{50} by several orders of magnitude (Senkevich, Koonin, and Buller, 1994b). Functional p28 protein was found necessary for ECTV replication in some primary murine macrophages (Senkevich, Wolffe, and Buller, 1995). By analogy, if a functional p28 gene is necessary for efficient virus replication in alveolar macrophages and subsequent spread to, and replication in, the draining hilar lymph node following a respiratory infection, this might explain the reduced dermal VACV titres as compared to RPXV-UTR in the hilar lymph node and internal organs of infected rabbits (Westwood et al., 1966).

The protein product of RPXV-UTR 180, which is predicted to be a 791 aa ortholog of VARV-BSH B18R, contains three ankyrin repeat motifs (PROSITE database PDOC50088; www.expasy.ch). This is an interesting protein because evidence is accumulating that ankyrin repeat motifs mediate protein-protein interaction events, such as those between integral membrane and cytoskeletal proteins (Lambert et al., 1990; Lux, John, and Bennett, 1990). Moreover, poxvirus proteins containing ankyrin repeat motifs are thought to influence virus host range and pathogenesis (Shchelkunov, Blinov, and Sandakhchiev, 1993; Shchelkunov et al., 1998). Orthologs of RPXV-UTR 180 are present in many of the OPVs but are fragmented in VACV (Fig. 5). A notable exception among the virulent OPVs is ECTV that lacks an ortholog of RPXV-UTR 180 due to a series of small deletions that shift the reading frame. However, both ECTV and CPXV

contain a paralog of this gene at the left end of the genome which is predicted to encode a protein that has approximately 46% amino acid identity; thus, it is possible that this ankyrin repeat motif containing gene may still be important for virulence in RPXV and that the ECTV-MOS 005 functions to complement the loss of the RPXV-UTR 180 ortholog in ECTV (Fig. 5). The C-terminal region of RPXV-UTR 180 also contains some similarity to the F-box (PROSITE database PDOC50181; www.expasy.ch) motif. This protein motif is believed to play a general role in protein-protein interactions and functions in the association of the Skp1-cullin-F-box protein ligase complexes; it is associated with ubiquitination and degradation of several proteins (Bai et al., 1996).

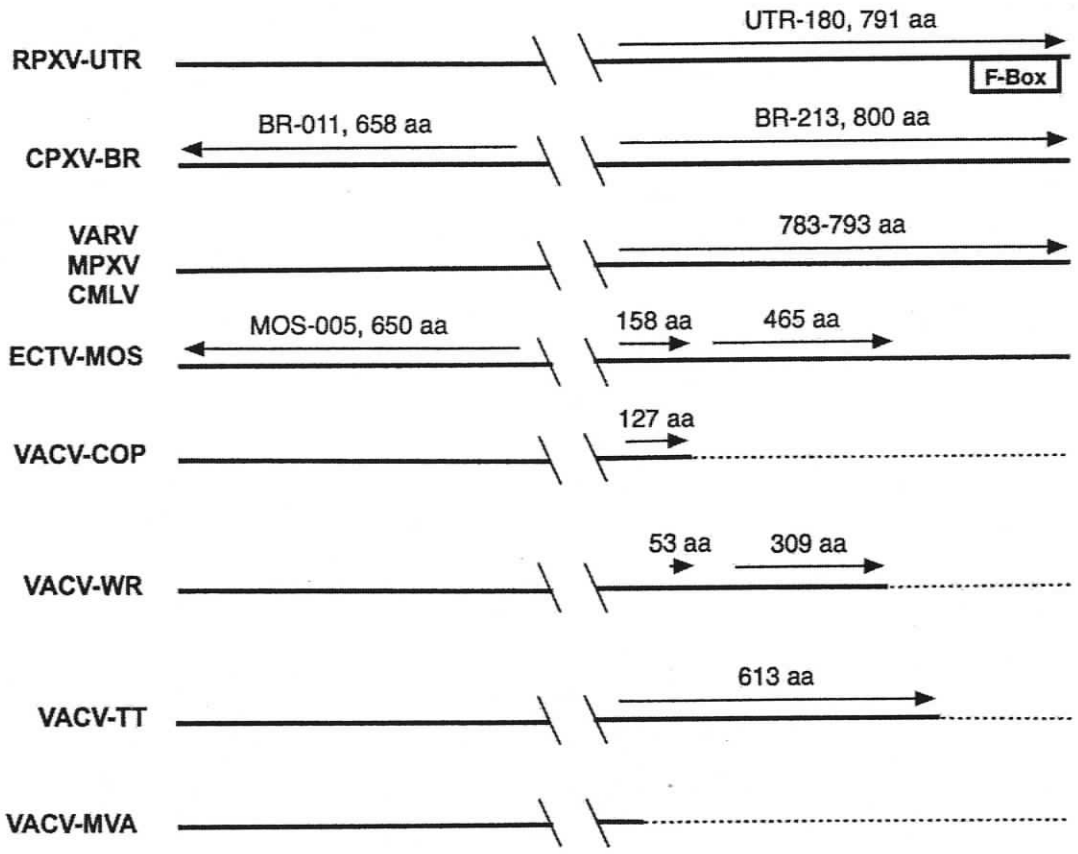


Figure 5 Organization of the RPXV-UTR-180 orthologs, paralogs and gene fragments at left and right ends of OPV genomes. -\ \- indicates the omitted central portion of the genomes. Dotted lines indicate sequences deleted in VACV genomes. Length of predicted proteins for potential genes and ORFs are indicated. The region shown for the left end of the genome is approximately 10-15 kb from the left hairpin.

The RPXV-UTR 180 gene is also interesting because it contains 719 nucleotides (from 186,989 to 187,708; Fig. 5) that are not present anywhere in the genomes of any of the 4 sequenced VACV strains; this encodes the C-terminal end of the RPXV-UTR 180 protein. Although an orthologous region is present in other sequenced OPVs, they possess minor, but significant differences that exclude the possibility that this is a contaminating laboratory sequence arising from the processes of DNA sequencing. This finding has two implications. First of all, the F-box domains in these proteins interact with cellular ubiquitin ligase complexes and thereby direct the ubiquitin ligase complexes bound to ankyrin repeats (Mercer, Flming, and Ueda, 2005). This gene should not be functional in any currently sequenced VACV although it remains as fragments in some VACV (Fig. 5). Second, it appears that RPXV is not derived from known VACV isolates, but was probably present in the uncloned population of VACV in use at the time of isolation and that it was selected naturally by its virulence in rabbits.

RPXV-UTR 001/184 are ITR genes that belong to the chemokine binding protein family of poxvirus proteins. Although the promoter and gene are very similar in all OPVs, in several VACVs a small deletion close to the 5' end of the gene generates a frame shift mutation that results in the synthesis of a severely truncated protein (Fig 6). This 7.5 kDa gene product gives its name to the well characterized p7.5 early promoter in VACV-WR (Wittek et al., 1980a; Wittek et al., 1980b; Wittek and Moss, 1980). Unexpectedly, deletion of the RPXV CBP gene did not lead to attenuation in mice or rabbits, but rather to slight shortening of the time before onset of illness; it was suggested that this gene

might enhance spread of the virus by lessening symptoms early in the infection process (Martinez-Pomares, Thompson, and Moyer, 1995). Similarly, insertion of the VACV-Lister gene for this CBP into VACV-WR reduced virulence in mice (Reading, Symons, and Smith, 2003). This phenotype is not without precedence, deletion of the IL-1 binding protein gene resulted in greater disease symptoms due to increased levels of IL-1 (Alcami and Smith, 1992). Thus, it is reasonable to still consider the CBP as a virulence factor.

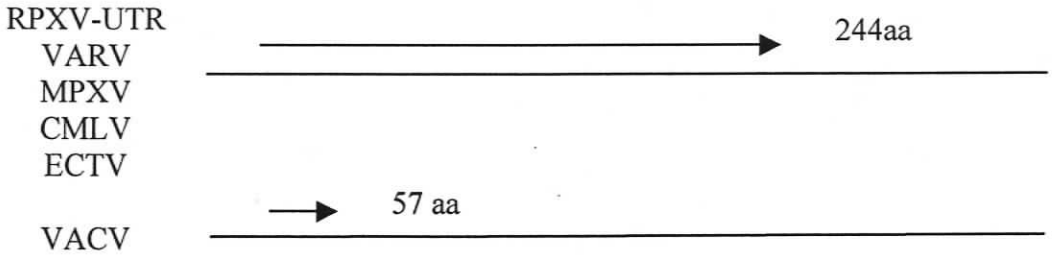


Figure 6 CBP of RPXV-UTR-001/184 orthologs and gene fragments in OPV genomes. Dark lines indicate OPV genomes. Arrows show the gene encoding CBP family in RPXV-UTR, VARV, MPXV, CMLV and ECTV, and the severely truncated fragment of CBP in VACV. Length of predicted proteins for potential genes and ORFs are indicated.

It is also interesting that RPXV-UTR 134 is only full length in the WR strain of VACV and VARV (Fig. 7). The product of RPXV-UTR 134 is an ortholog of the OPV structural protein (P4c) that directs IMV particles into A-Type inclusions (McKelvey et al., 2002). The general conservation of this gene in a number of OPVs that do not produce a full length A-Type inclusion protein suggests that both the P4c proteins and the partial A-Type inclusion proteins are probably still be providing a selectable, but currently unknown, advantage to these viruses.

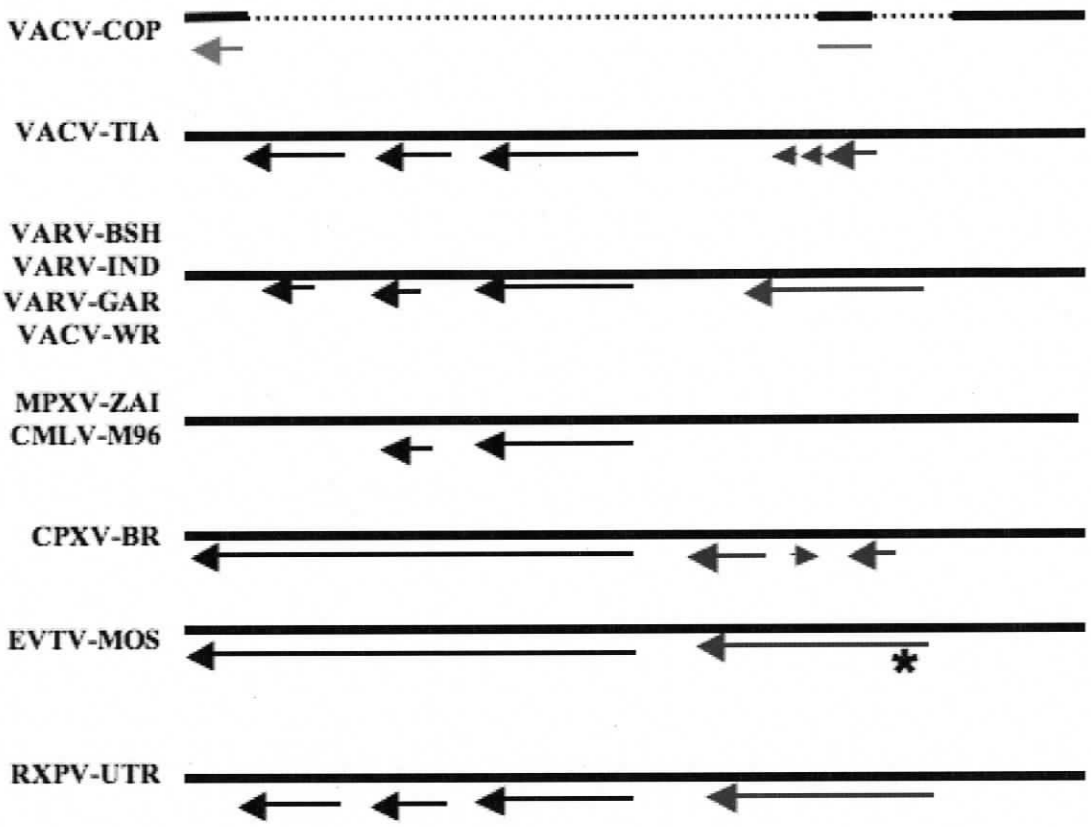


Figure 7. A Type inclusion protein gene family in OPVs genomes. Dark line: DNA sequence of genome; dash line: DNA deletion; * stop codon; blue arrow: counterparts of IMV surface protein/A type inclusion of RPXV-134 in OPVs genome; dark arrow: counter parts of A type protein of CPXV-BR-158; green line and arrow: the remnant of A type inclusion protein in VACV-COP.

3.1.4 Fragmented Regions of RPXV Genome

In annotating the RPXV genome, a number of small ORFs that are clearly fragments of larger genes present in other OPVs were intentionally excluded. This is important because those fragments are very unlikely to be functional. However, for completeness, 12 regions are described that clearly correspond to disrupt ORFs; these are named as fragmented regions I to XII (Table 3; Fig. 1).

Mutations in these regions were mapped by comparing the RPXV DNA sequence to the protein sequence of the longest OPV orthologs using the NAP program, a tool for alignment of nucleotide and amino acids. It is very unlikely that these 12 regions generate functional gene products because the ORFs contained within them are all significantly truncated with respect to their functional orthologs. However, further work is required to confirm this loss of function.

Table 3 Regions in the RPXV-UTR genome corresponding to fragments of annotated ORFs in other OPVs. *Regions present in the ITRs of RPXV-UTR

RPXV-UTR				Longest OPV Otholog		Annotation
Region	Start	Stop	Significant ORF fragments (bp)	Gene name (bp)	Function/Motif	OPVs with intact gene
I/XII*	2,870/193,907	3,835/194,859	192 369	CPRXV-BR-005 (1065)	TNF-alpha receptor-like	I: CPXV, MPXV, ECTV XII: CPXV, CMLV, MPXV, ECTV,VARV
II/XI*	3,899/192,162	5,570/193,833	234 330 342 450	CPXV-BR-006 (1857)	Ankyrin repeats	II: CPXV, CMLV, MPXV, ECTV XI: CPXV, CMLV, MPXV, ECTV, VARV
III/X*	5,687/190,073	7,605/192,045	492 1158	CPXV-BR-008 (2016)	Ankyrin repeats	III: CPXV, CMLV X: CPXV
IV	15,338	17,304	216 234 1230	CPXV-BR-025 (2004)	Host range	CPXV, MPXV
V	33,549	34,339	255 366	ECTV-MOS-024 (831)	Putative monoglyceride lipase	CPXV, MPXV,ECTV
VI	139,433	143,226	684 702 2178	CPXV-BR-158 (3852)	ATI protein	CPXV, ECTV
VII	162,502	163,510	255 363 381	VACV-COP-A51R (1002)	Unknown	All other sequenced OPVs
VIII	169,747	171,243	375 660	CPXV-BR-197 (1515)	Unknown	CMLV, CPXV,ECTV, MPXV
IX	180,049	181,031	405 624	ECTV-MOS-163 (984)	IL-1 beta receptor-like	ECTV, CPXV, VACV, MPXV

All but two of the fragmented regions in RPXV-UTR correspond to regions that are also fragmented in other VACV genomes. Fragment region VII of RPXV-UTR is a disrupted version of VACV-COP A51R; the function of this gene is unknown but it is present in all other OPVs. The mutation that disrupts the RPXV ortholog of VACV-COP A51R was very clear in the sequence trace data; since DNA sequencing was performed in two directions using multiple pooled PCR products as the DNA sequencing template we are confident that this and other mutations that disrupt coding sequences are not from PCR mutations or sequencing errors.

The fragmented hemagglutinin (HA) gene was annotated as RPXV-UTR 163 because it represents approximately the C-terminal two-thirds of the full length protein, however, this is additionally annotated as "fragmented" in the VOCs database (www.poxvirus.org). The RPXV HA gene frame-shifts because of an additional adenine residue after a run of 6 adenines; the gene was re-sequenced and analyzed using 2 different sequencing machines and software packages to remove any systematic error. Fragmentation of the HA gene was also confirmed (R. Roper, personal communication) by visualizing the plaque phenotype of RPXV-UTR in which the cells fuse at the edges of the plaques (Ichihashi and Dales, 1971). Thus, although the gene encoding hemagglutinin is conserved in all other OPVs, the sequencing and experimental data indicates that this isolate of RPXV is indeed HA negative.

3.2 MPXV Study

The MPXV study was divided into four parts. In the first part, the virulence difference between the West African isolate of MPXV-COP and the Central African isolate of MPXV-ZAI-V79 in monkey was tested (by collaborators). In the second part, three West African MPXV isolates were sequenced, assembled, annotated and analyzed phylogenetically. In the third part, MPXV-SL-V70, a West African MPXV representative isolated from a human patient, was compared against MPXV-ZAI-96, the prototypic Central African isolate. In the last part, collaborators tested the activity of the virulence gene MPXV-ZAI D14L which is thought to be most likely responsible for the enhanced virulence of the Central African isolate over the West African isolates (MPXV involved in this research were listed in Table 1 in the Material and Methods).

3.2.1 Testing for the Virulence Differences between West African and Central African isolate of MPXV

The collaborator in the United States Army Research Institute of Infectious Diseases tested the virulence of the West African (MPXV-COP-58) and Congo basin (MPXV-ZAI-V79) isolates by aerosol infection of cynomolgus monkeys with high and low doses of virus (Table 4). The West African isolate caused no deaths and little morbidity, whereas infections with the Congo basin isolate resulted in severe morbidity at both high and low doses and uniform mortality at the high dose. To confirm if this virulence

difference was due to genetic differences in virus-encoded virulence genes, Dr. Nanhai Chen in Dr. Mark Buller's Lab performed the comparison of the kinetics of MPXV-COP-58 and MPXV-ZAI-V79 to see if the replication and cell-to-cell spread of both viruses occurred in tissue culture with similar rate. Under conditions of a single-cycle infection, the yield of cell-associated and cell-released virus at all time points in MPXV-COP-58 and MPXV-ZAI-V79 infected BSC-1 cultures was not statistically different according to the standard t-test that is the most common biostatistics method for quantity data (Belle et al., 2004) (Fig. 8; $p>0.05$, with assumed equal variances). The diameter and character of the plaques was identical (Fig. 8; inset), and the release of EEV from infected cells as determined by the comet assay was also similar (Buller, unpublished; Payne, 1980). This data suggests that mutational differences between the West African and Congo basin isolates of the Central African do not affect the production of IEV virus in tissue culture, and is consistent with the genetic basis for differences in virulence residing in non-essential virulence genes (Appendix B).

In an attempt to identify the genes responsible for the difference in virulence, three West African isolates (MPXV-COP-58, MPXV-SL-70 and MPXV-WRAIR-61) were sequenced, and then compared to the genomic sequence the Central African isolate (MPXV-ZAI-96).

Table 4 Aerosol Infection of Cynomolgus Monkeys with West Isolates and Congo Basin Isolates Monkeypox. [1] An aerosol route of challenge was used, as it is probably the major route for person-to-person transmission of MPXV. The aerosol challenge particle size was 1-3 μm. [2] exanthem, enanthem, cough, depression. [3] late enanthem ~10 days. 3 monkeys (1.6-4.7 kg) were used per group.

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

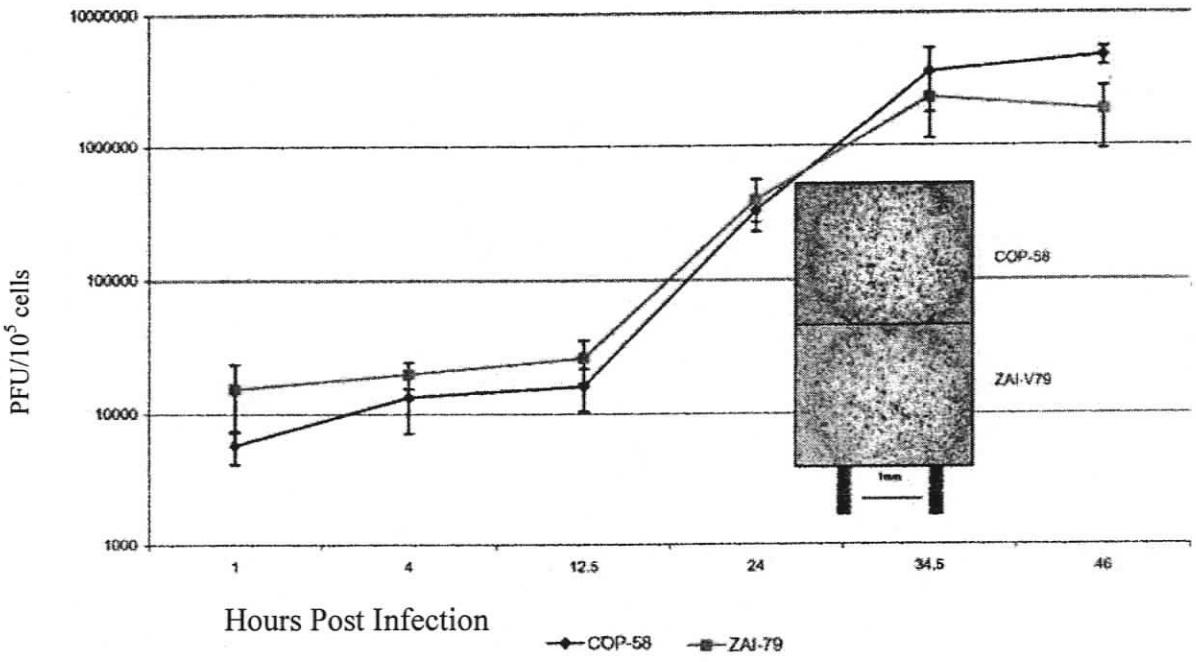


Figure 8 Comparison of single-cycle replication yields of MPXV-COP-58 and MPXV-ZAI-V79. Monolayer cultures of BSC-1 cells were infected with MPXV-COP-58 or MPXV-ZAI-V79 at approximately 1 PFU/cell. At 1, 4, 12.5, 24, 34.5 and 46 hrs post-infection 4 cultures were harvested for each virus. Cells were scraped into the culture supernatant, frozen and thawed 3 times and infectivity was measured by plaque assay on BSC-1 monolayers. Plaque titers are presented as means with error bars indicating 1 standard deviation of the mean. The inset shows a typical MPXV-COP-58 and MPXV-ZAI-V79 plaque stained at 4 days post-infection.

3.2.2 Sequencing of the West African MPXV Isolates and Phylogeny Analysis

3.2.2.1 Sequencing of West African Isolates.

Single contiguous sequences of 198,756, 199,195, and 199,469 bp were obtained for MPXV-SL-V70, MPXV-WRAIR-61, and MPXV-COP-58, respectively. The palindromic hairpin terminal loops (~80 bp) at each end of the genomes were not sequenced. The first nucleotide of each genomic sequence of MPXV-SL-V70, MPXV-WRAIR-61 and MPXV-COP-58 are equivalent to nucleotide 160, 155 and 149 of the genomic sequence of VACV-COP, respectively. This position was 36, 30 and 25 nucleotides, respectively, beyond the end of the first primer used to amplify the sequencing templates.

3.2.2.2 Genome Overview and Classification of the West Africa MPXV Isolates

Like other poxviruses, ITRs are detected at each end of the Western African MPXV genomes. The ITRs are identical but oppositely oriented sequence at the two ends of the genome, and therefore the genes located in ITR are duplicated (Moss, 2001). The ITRs of the MPXV-SL-V70 genome account for 8,573 bp at each end of the genome (Fig. 9), leaving a unique region of 181,610 nucleotides. The ITRs of MPXV-SL-V70, MPXV-WRAIR-61, and MPXV-COP-58 are longer than those in MPXV-ZAI-96, the difference results in the presence of 6 genes in the ITRs of the West African isolates compared to 4 genes in the ITRs of MPXV-ZAI-96. The 6 genes include the genes in the ITRs plus 2

non-ITR genes from the right end of the MPXV-ZAI-96 genome, thus the genomes are organized almost identically, except for the ITR diploid gene portions, which are larger in MPXV-SL-V70. It is not known how the ITRs are created or maintained during replications, but it is well known that ITRs vary among isolates. The 177 putative genes were annotated (Appendix B), including 46 unknown function genes, 43 genes that are related to host range or the evasion of the host defense, and 88 genes that are thought to be essential for virus replication in standard tissue culture cell lines (e.g. functions mainly necessary for transcribing mRNA, replicating the genomic DNA, and assembling infectious virions) (Table 5)

Table 5 Summary of the West African MPXV annotated ORFs

Function of predicted genes	Numbers
Essential genes for virus replication in standard cell lines	88
Genes related to host range and the evasion of host defense	43
Genes with unknown function	46

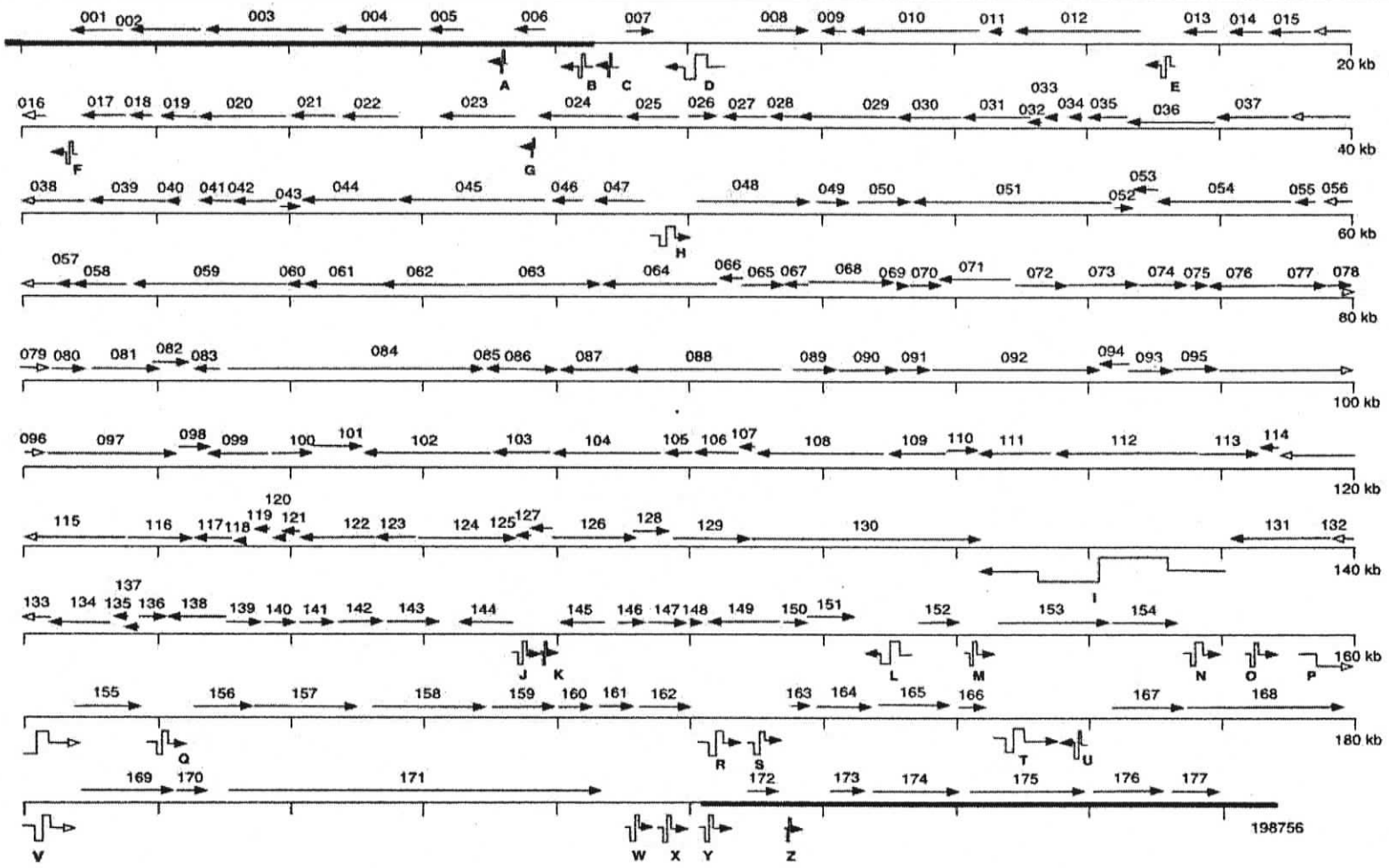


Figure 9 Physical map of the MPXV-SL-V70 genome. Predicted genes are numbered and shown as straight arrows; regions containing fragments of larger genes in other OPVs are shown with staggered arrows to represent frame changes and are labeled A-Z. Open arrowheads indicate an ORF is split over 2 lines of the diagram. Scale is shown in kilobases. The thick line represents the ITR.

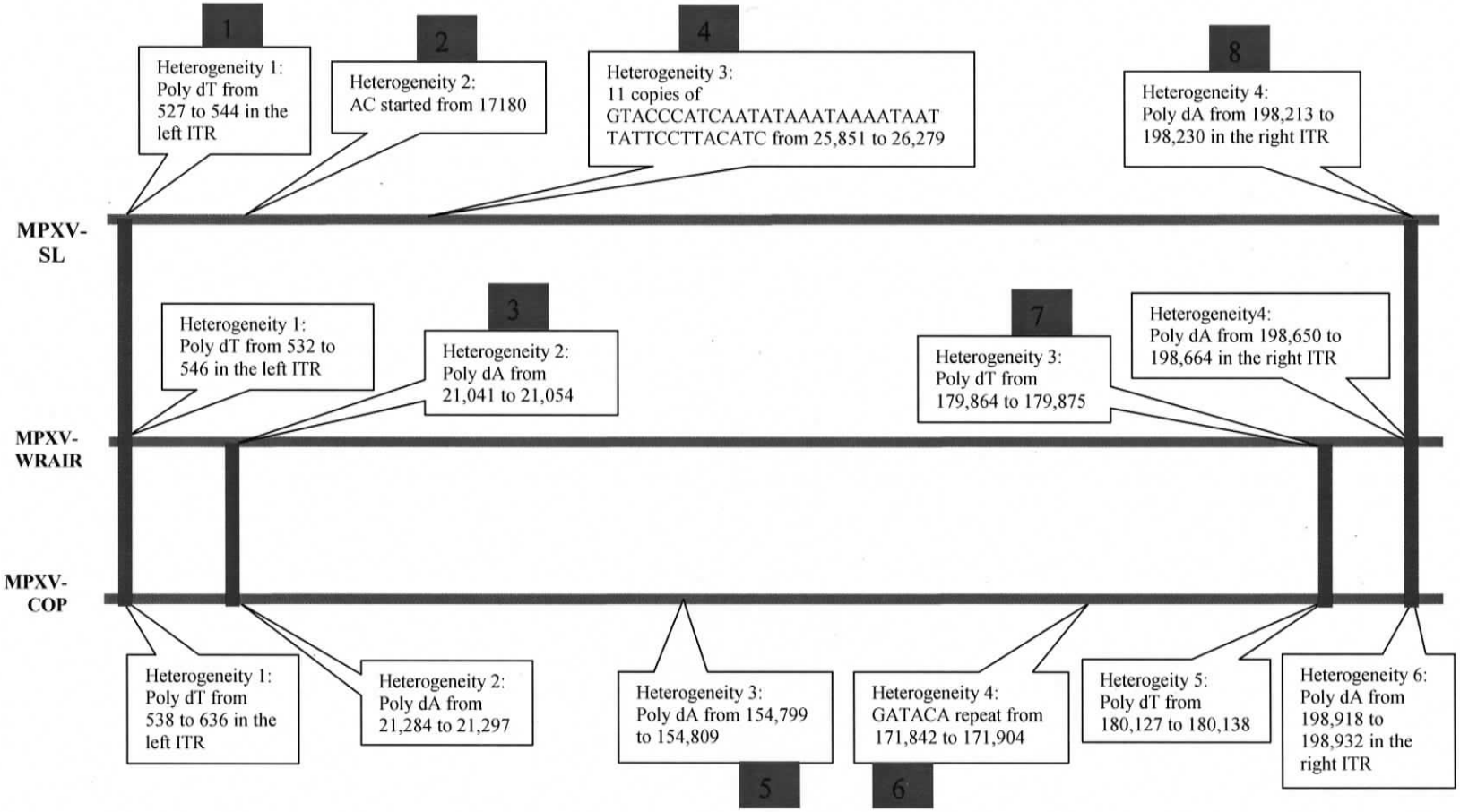


Figure 10 Heterogenity in three West African MPXV isolates. Blue bars show the same heterogeneity position in the corresponding region of the genomes of different isolates. Green line: genome of different isolates. Site 1 and 8 exist in the genomes of all three isolates; site 3 and 7 exist in MPXV-WRAIR-61 and MPXV-COP-58; site 2 and 4 only exist in the genome of MPXV-SL-V70; site 5 and 6 only exist in the genome of MPXV-COP-58.

The three sequenced West African isolates, particularly the MPXV-WRAIR-61 and the MPXV-COP-58, are very similar (see discussion later) and it is possible that some of these differences are due to minor sequencing errors (we were unable to check genomes sequenced by other groups). To clarify this, eleven positions within the MPXV genome were identified to help aid in the classification of isolates. Eight of these sites show DNA sequence micro-heterogeneity in the West African MPXV isolates (Fig. 10).

MPXV-SL-V70 has a DR-like sequence (AACTAACTTATGACTT) + (AACTAACTTATGACT) which is present in the left (4,628 to 4,658) and right (194,099 to 194,129) ITR. 12.5, 19 and 1.7 copies of the AACTAACTTATGACTT element appear at the corresponding locations in MPXV-WRAIR-61, MPXV-COP-58 and MPXV-ZAI-96 genomes, respectively, but no such repeats appear in other reported OPV genomic sequences.

The ITRs of the West African MPXV-SL-70, MPXV-WRAIR-61 and MPXV-COP-58 isolates also contain two copies of a 50 bp region (CCATCAGAAAGAGGTTTAATATTTTTGTGAGACCCATCGAAGAGAGAAAG) separated by 20 bp of unique sequence; this 50 bp element at position 28-77 and 98-147in MPXV-SL-V70 is a truncated version of a highly repeated 70 nucleotide DR element of VACV-COP (nucleotide position 973-2849 of VACV-COP). However, it is different in the genome of the Central African isolate of MPXV-ZAI-96, in which nucleotide 41 of the second copy is C rather than an A (...cccatcgaCgagagaaag).

A poly dT tract is located in the non-coding region of the left ITR of MPXV-SL-V70 between nucleotides 527-544. Of 11 clones sequenced, four were dT_{17}, three dT_{18}, two dT_{19} and one each dT_{14} and dT_{16}. As expected, the reciprocal dA tract in the right end ITR (198,213 to 198,230) was similarly heterogeneous; of the twelve clones sequenced, five were dA_{18}, four dA_{19}, two dA_{17} and one dA_{16}. Thus the longest common tract of dA/T_{18} was reported for the ITRs of the MPXV-SL-V70 sequence deposited into GenBank. The corresponding regions in the MPXV-WRAIR-61, and MPXV-COP-58 isolates are also heterogeneous, and both were reported to be dA/T_{15}; dA/T_7 is found at the corresponding region of MPXV-ZAI-96.

Another heterogeneous region was found at position 17,180 in the MPXV-SL-V70 genome. Of ten clones sequenced, only two contained a two-nucleotide "AC" deletion. Thus the "AC" was included in the sequence that was deposited into the GenBank database. The corresponding regions of MPXV-WRAIR-61, MPXV-COP-58, and MPXV-ZAI-96 isolates also contain "AC" at this position.

In MPXV-WRAIR-61, a tract of poly dA appears between nucleotides 21,041 to 21,054; this is closely followed by the stop codon of ORF 017, an ortholog VACV-COP-C1L. Of eleven clones sequenced, four clones were dA_{13}, six clones were dA_{14} and one clone was dA_{15}. Our GenBank deposit shows a tract of dA_{14}. At the corresponding position of

MPXV-SL-V70, MPXV-COP-58 (heterogeneous in MPXV-COP-58) and the Central African MPXV-ZAI-96 there is dA_9, dA_{14} and dA_8, respectively.

A 39-nucleotide repeat element (GTACCCATCAATATAAATAAAATAATTAT TCCTTACATC) appears at nucleotide 25,851 to 26,279 of the MPXV-SL-V70 genome, 18 nucleotides following the stop codon of ORF 23, an ortholog of SPI (VACV-COP K2L). We sequenced 8 clones; 5 contained 11 copies, and 3 contained 10 copies of the repeat. Our GenBank deposit shows 11 copies. The corresponding regions of the MPXV-WRAIR-61, MPXV-COP-58, and the Central African MPXV-ZAI-96 genomes contain 7, 7 and 1 copy, respectively.

In the MPXV-COP-58, a heterogeneous tract of poly dA appears at 154,799 to 154,809, 15 nucleotides upstream of the start codon of ORF 153, an ortholog of VACV-COP A50R (DNA ligase). Of the 11 clones sequenced, 10 clones were dA_{11}, and 1 was dA_{10}. Our GenBank deposit shows a sequence of dA_{11}. The corresponding regions of MPXV-SL-V70, MPXV-WRAIR-61 and MPXV-ZAI-96 contain dA_9, dA_{11} and dA_9, respectively.

In the MPXV-COP-58, a 6-nucleotide repeat element "GATACA" appears at 171,842 to 171,904, that is located upstream of the 5' end of ORF 163, the ortholog of VACV-COP 11R (unknown function). Of the 12 clones sequenced, 7 and 5 clones contained 10.5

copies and 9 copies of the repeat element. Our GenBank deposit shows 10.5 copies of the repeat element. The corresponding regions of MPXV-SL-V70, MPXV-WRAIR-61, and MPXV-MPXV-ZAI-96 contain 6.5, 10.5 and 7.5 copies of the repeat element, respectively.

In the MPXV-WRAIR-61, a heterogeneous tract of poly dT appears at 179,864 to 179,875, 21 nucleotides following the stop codon of ORF 168, an ortholog of the fragmented VACV-COP-B20R. We sequenced 10 clones; 2 were dT_{11}, 6 were dT_{12}, 1 was dA_{13} and 1 clone dT_{14}. Our GenBank deposit shows a tract of dT_{12}. The corresponding regions of MPXV-SL-V70, MPXV-COP-58 (heterogeneous in MPXV-COP-58), and MPXV-ZAI-96 contain dT_{10}, dT_{12}, and dT_{10} repeats, respectively.

An unusual repeat of 7 tandem copies of CATTATATA (180,896 to 180,958) appears in the genome of MPXV-SL-V70 compared with 37, 27, 16 identical copies of the sequence in the MPXV-WRAIR-61, MPXV-COP-58, and MPXV-ZAI-96 genomes, respectively. In most other OPVs, there is only one copy of this repeat element.

These differences in the number and position of minor repeat elements and a series of poly dA/dT tracts among the genomes confirm that the MPXV-COP-58, MPXV-WRAIR-61 and MPXV-SL-V70 are very similar, but different isolates. As

representatives of MPXV from West African, those 3 West African isolates were compared to the Central African isolate MPXV-ZAI-96.

3.2.2.3 Comparison of the genes of the West African isolates MPXV-SL-V70, MPXV-COP-58, and MPXV-WRAIR-61

There are no differences in the set of genes predicted for the three West African isolates. To further examine if other minor sequence were likely to result in differences that might interfere with the conclusions derived from the animal experiments, the protein coding regions of the genomes of the West African isolates were compared in greater detail.

In the protein coding regions, 77 substitutions, insertions or deletions were found in 53 ORFs with pair-wise comparisons between MPXV-SL-V70 and MPXV-COP-58; 22 of the mutations result in aa changes. Four mutations result in conservative aa substitutions and 18 mutations result in non-conservative mutations (Table 6). Our analysis predicts length changes due to repeat elements in 7 MPXV-COP-58 ORFs: 014, 023, 029, 114, 131, 163, and 169. Compared to MPXV-COP-58 014, an ortholog of VACV-COP C6L (unknown function) has an $(Asp)_5$ at the C terminus instead of Asp_9. MPXV-COP-58 023, an ortholog of VACV-COP K2L (SPI-3), has an Asp_3 at the N-terminus in place of the $(Asp)_4$. MPXV-COP-58 029, an ortholog of VACV-COP F3L (kelch-like protein of unknown function), has EWNGK at the C terminus, instead of VNNFEIK. MPXV-COP-58 114, an ortholog of VACV-COP A9L (IMV, membrane protein), has $(Ser+Asn)_5$ near the C terminus in place of $(Ser+Asn)_8$. MPXV-COP-58 131, an ortholog of VARV-BSH-

75 (ATI factor), has an $(Asp)_{10}$ in the middle of the ORF in place of $(Asp)_{16}$. This ORF is fragmented in ECTV suggesting its function is not important in all pathogenic OPVs. MPXV-COP-58 163, an ortholog of VACV-COP 11R (unknown function), has $(Asp+Thr)_9$ near the C terminus in place of $(Asp+Thr)_6$. And finally, MPXV-COP-58 169, an ortholog of VACV-COP C12L (SPI1), has $(Ile+Ile+Tyr)_{37}$ near the N-terminus in place of $(Ile+Ile+Tyr)_6$. Table 6 also summarizes a pair-wise comparison between isolates MPXV-SL-V70 and MPXV-WRAIR-61 (last column, grey shading). Of the 11 differences noted from the MPXV-SL-V70 and MPXV-COP-58 comparison, 4 mutations are not present, 3 mutations differ in the length of the same indel, and 4 mutations are unique substitutions, with 3 of the 4 being silent mutations. Careful examination of the structure and function (where known) of each affected ORF does not support biological consequences of any of the mutational differences among MPXV-SL-V70, MPXV-COP-58, and MPXV-WRAIR-61.

In conclusion, the examination of the minor sequence differences among three West African MPXV isolates (MPXV-SL-V70, MPXV-WRAIR-61 and MPXV-COP-58) does not predict any biological consequence for these changes, and therefore, for the comparison to MPXV-ZAI-79, the West African isolates MPXV-SL-V70 and MPXV-WRAIR-61 should have the same attenuated phenotype in cynomolgus monkeys as MPXV-COP-58 (Table 4).

Table 6. Comparison of nucleotide differences between MPXV-SL-V70, MPXV-COP-58, and MPXV-WRAIR-61. * conservative aa change: aa changes among the aa that have the similar structure: small and non-polar (G, C, T, A, S), small and polar (E, D, N, Q), large and non-polar (V, I, M, F, L), or large and polar (K, H, R, W, Y). Grey shading row: ORFs that have a different mutations in MPXV-SL-V70/MPXV-COP-58 compared to that of MPXV-SL-V70/MPXV-WRAIR-61.

MPXV-COP-58 Mutations as compared to MPXV-SL-V70								
ORF of MPXV-SL-V70	Length	Aligned Length	Type of mutations		Consequence of mutations			Presence of noted MPXV-COP-58 mutations in MPXV-WRAIR-61
			Subs	Indel	aa Changes	Silent Changes	Length change of ORF (bp)	
2	1050	1050	1		1 (Leu to Ser)			+
3	1773	1774	3		1 (Ala to Glu)	2		+
4	1314	1314	3		1 (Glu to Ala)	2		+
7	429	430	2		1 (Ala to Asp)	1		+
10	1983	1983	1			1		+
14	480	465		1			12	9 bases change in ORF length
16	951	951	1		1 (Asn to Asp)*			+
17	645	645	1		1 (Arg to Cys)			+
19	534	534	1		1 (Asp to Gly)			+
20	1341	1342	1			1		+
23	1125	1128	1	1		1	-3	+
26	450	450	3		1 (Val-Ala)	2		+
28	456	456	1			1		+
29	1479	1489		1			1	+
31	1032	1033	1		1 (Leu to Ser)			-
37	1065	1065	1			1		+
38	1908	1908	1		1 (Asn to Ser)*			+
44	1440	1440	1		1 (Ile to Val)*			+
48	1704	1704	1			1		+
50	822	822	1			1		-
61	1149	1149	1			1		+

63	2031	2031	1		1 (Pro to Ser)			+
67	375	375	1			1		+
70	498	498	1		1 (Leu to Val)*			+
81	1002	1002	3		1 (Thr to Ile)	2		+
83	402	402	1		1 (Asn to His)			+
84	3861	3862	2			2		+
88	2388	2388	1			1		+
89	633	642	1			1		+
90	945	945						1 mutation (Ser to Asn)*
92	2538	2538	1			1		2 silent mutations
93	702	702	1			1		+
94	441	441	1			1		+
95	657	657	2			2		+
108	1935	1935	1			1		+
114	339	321	1	1	1 (Ser to Leu)		18	+
115	2676	2676	1			1		2 silent mutations
123	615	615	1			1		+
124	1479	1479						1 silent mutation
127	348	348	1		1 (Val to Met)*			+
131	1521	1500	1	1		1	21	-
144	834	834	1			1		+
153	1680	1680	2			2		+
157	1518	1518	1			1		+
158	1695	1695	2			2		+
159	954	954	1		1 (Asn to Ser)*			+
163	98	106	1	1		1	-18	No subs; 24 base change in ORF Length
165	1035	1035	1			1		-
166	450	450	1		1 (Arg to Gly)			+
167	1059	1059	1			1		+
169	1194	1374	2	1		2	-180	-270 base length change of ORF length
171	5643	5643	2			2		+
174	1314	1314	3		1 (Glu to Ala)	2		+
175	1773	1774	3		1 (Ala to Glu)	2		+
176	1050	1050	1		1 (Leu to Ser)			+
Total			70	7	22	48	7	

3.2.2.4 Phylogeny Analysis

Based upon multiple nucleic acid sequence alignments of the core conserved genomic region of each OPV species, the evolutionary relationships between these viruses were determined and are shown in Figure 11. The phylogenetic tree indicates that the three West African isolates represent a clade that is distinct from the Central African isolate of MPXV-ZAI-96.

To further examine the relatedness of the MPXV isolates, MPXV-SL-V70, the representative of the West African isolate, was compared with the Central African isolate MPXV-ZAI-96, the sole Congo basin (Central Africa) group member whose genomic sequence is available in GenBank (Shchelkunov et al., 2001; Shchelkunov et al., 2002). We chose MPXV-SL-V70 as the prototypic West African isolate because it was isolated from a case of human monkeypox in Sierra Leone (Foster et al., 1972). Figure 12A shows a graphic representation of the substitutions, insertions and deletions (indel) observed between the aligned genomes. MPXV-SL-V70 is clearly much more closely related to isolates MPXV-WRAIR-61 and MPXV-COP-58 than to MPXV-ZAI-96.

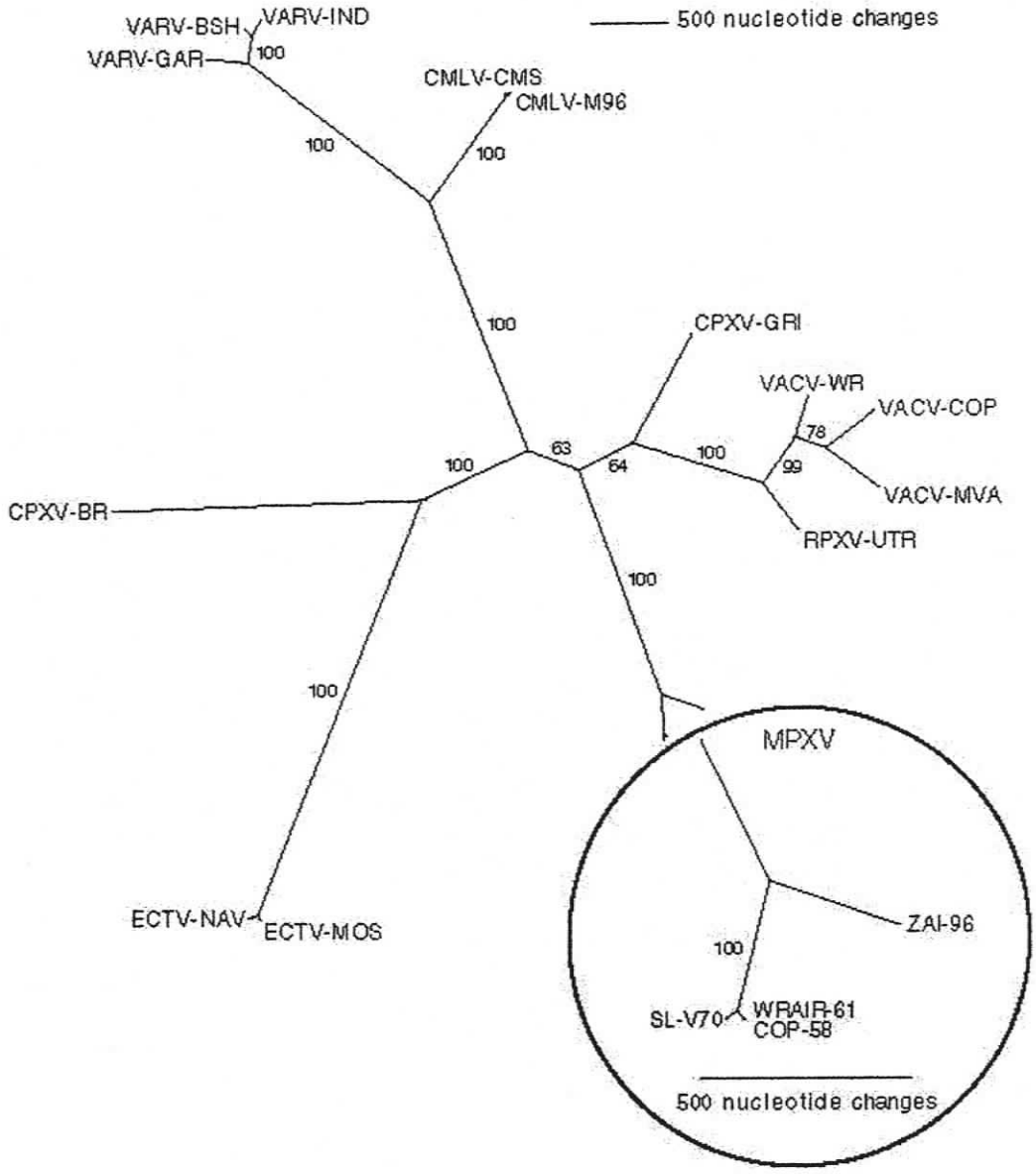
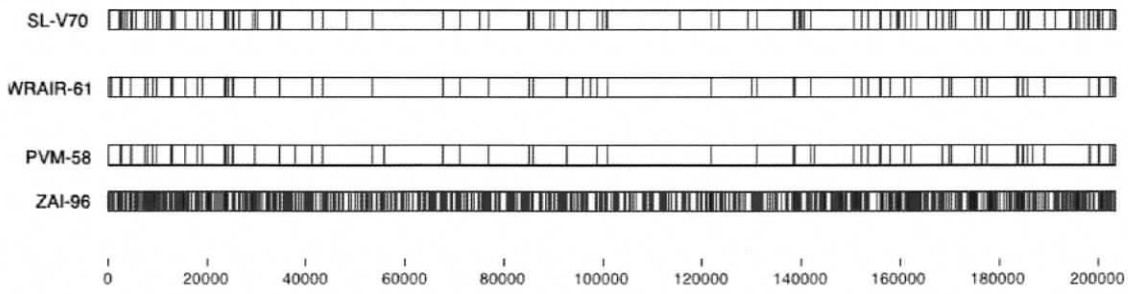


Figure 11 Phylogenetic tree of MPXVs and other OPVs. OPV phylogenetic predictions based upon the multiple nucleic acid sequence alignments of the core genomic region of each representative orthopoxvirus species, strain or isolate. Bootstrap resampling confidence values on 1,000 replicates are displayed at each branch point. Branch lengths are proportional to the number of nucleotide changes.

A



B

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

Figure 12 Genomic comparison of West and Congo basin isolates of MPXV. (A) CLUSTALW software was used to align the genomes of SL-V70, COP-58, WRAIR-61 and ZAI-96 and the alignment was manually optimized using Base-By-Base. Each mis-matched base was identified as a substitution (blue bar), deletion (red bar) or insertion (green bar) relative to a consensus; blue bars in all genomes indicate no consensus. Insertions and deletions were counted as one mis-match regardless of size. The scale is such that several substitutions in close proximity may generate a single blue bar. (B) Summary nucleotide identity comparisons: upper (grey) =gap number (segments) / total gap length; lower=number substitutions / number identical (non-gap) residues/ percent difference (includes number of gaps).

In addition, the comparison between MPXV-COP-58 and MPXV-WRAIR-61 also shows they are more closely related to each other than to MPXV-SL-V70, but are distinguished from each other by the number and position of minor repeat elements and a series of poly dA/T sequences distributed along the genome discussed above; this information may be useful for distinguishing closely related isolates in molecular epidemiological studies. Figure 12B summarizes percent identity values for individual pairwise comparisons. The three West African isolates show a mean of 99.5% identity with MPXV-ZAI-96 as compared to 99.9% nucleotide identity among themselves. For comparison, we observe the following OPV intraspecies nucleotide identity values: 99.8% VARV (major strains Bangladesh-1975 and India-1967); 99.6% VARV (BSH-75, major strain and Garcia-1966, minor strain); 98.9% VACV (strains WR and COP); 99.6% ECTV (NAV and MOS isolates) and a 99.9% camelpox virus (M-96 and CMS isolates). This high level of identity among OPVs is consistent with the genes having a similar function in all OPVs.

In summary, there is significantly greater sequence diversity between the West African and Congo basin MPXV isolates than between three West African isolates indicating that the analyzed West African and Congo Basin (Central Africa) isolates belong to separate clades; this confirms and extends the MPXV RFLP studies of others (Douglass, Richardson, and Dumbell, 1994; Esposito and Knight, 1985; Mukinda et al., 1997).

3.2.3 Comparison of the West African Isolates and the Central African Isolates

Since there is no standardized method used by investigators to annotate poxvirus genomes, the first step in the process of comparing the genome sequences of MPXV isolates and VARV-BSH was to reannotate the MPXV-ZAI-96 and VARV-BSH-75 genomes as described in the Methods section.

3.2.3.1 Reannotation of the Genomes of MXPV-ZAI-96 and VARV-BSH-75

During the process of reannotating MPXV-ZAI-96 and VARV-BSH, we removed ORFs that are vestiges of conserved genes present in other poxviruses or small predicted ORFs on the non-coding strand. In the MPXV-ZAI-96 genome, we removed 14 fragmented ORFs that were previously annotated (D2L, D4L, D15L, D16L, D17L, D18L, C3L, A26L, A27L, B1R, B15L, B18R, K1R and R1R). The reannotated ZAI-96 genome contains 179 ORFs (Appendix B). MPXV-ZAI-96 was isolated during a human monkeypox outbreak in 1996 from scab material of a monkeypox patient residing in the Sankuru subregion, Kasdai Oriental, Zaire. The original annotation was described by Shchelkunov and colleagues (Shchelkunov et al., 2002).

We removed 15 fragmented ORFs that were previously annotated in VARV-BSH-75 genome (A29L, C7L, A28L, A27L, A26L, D17L, D16L, D10L, D9L, D8L, D1L, B20R, B19R, B11R and J6R). The reannotated VARV-BSH genome contains 176 ORFs. The original annotation was described by Massung and colleagues (Massung et al., 1994). These updated annotations are available from the POCsdb (www.poxvirus.org).

3.2.3.2 Comparisons of Transcription Regulatory Sequences among Western African and Central African Isolates

In order to assess the possibility that the virulence difference between the West African and Congo basin isolates was due to differences in the regulation of transcription, our collaborator, Dr. Chunlin Wang, compared the putative early, intermediate and late promoter core sequences and the early transcriptional termination sequence for all genes in the four MPXV isolates. This analysis detected only one mutation, an A to T change in the canonical core sequence (TATAT instead of TAAAT) of the late promoter for the MPXV-SL-V70 073 gene (ortholog VACV-COP G9R); this mutation is not conserved in two other Congo basin isolates and all ChPV, so it likely represents an error in the MPXV-ZAI-96 genomic sequence. While definitive conclusions await laboratory confirmation, it appears likely that changes in transcriptional regulation probably do not explain the difference in virulence between the West African and Congo Basin MPXV isolates.

3.2.3.3 Comparison of the genes of the West African Isolates and the Central African (Congo Basin) Isolate

The preceding data show that West African and Congo Basin MPXV isolates differ in virulence for cynomolgus monkeys, and that they are genetically distinct. To identify genes potentially responsible for the observed virulence difference, more extensive comparative analyses were carried out between West African MPXV-SL-V70 and Congo Basin MPXV-ZAI-96 isolates of MPXV. The general DNA difference between MPXV-SL-V70 and MPXV-ZAI-96 was shown by dotplot (Fig. 13). Within the MPXV-SL-V70 genome, we predict 171 functional unique genes, 26 non-functional fragmented ORF regions (Table 7), and small vestiges of an additional 10 ORFs (Fig. 9).

The MPXV-ZAI-96 genome is predicted to contain 175 unique genes (Appendix B) and 14 truncated ORFs, and differs little from the sequence of MPXV-ZAI-V79 isolated seventeen years earlier in the Congo basin, which suggests that isolates from this region share a similar evolutionary history and biology (Likos et al., 2005). MPXV-SL-V70 and MPXV-ZAI-96 share 170 unique orthologs that are on average more than 99.4% identical at the protein level.

A comparison of MPXV-SL-V70 and MPXV-ZAI-96 genomes found 170 indels for a combined length of 9,629 nucleotides and 852 nucleotide substitutions. 144 indels and 194 substitutions are in intergenic regions and fragmented genes. Five indels and 321 substitutions are distributed among 99 genes with functions thought to be essential for virus replication in standard tissue culture cell lines (e.g. functions mainly necessary for transcribing mRNA, replicating the genomic DNA, and assembling infectious virions). These genes are highly conserved in all sequenced OPVs and map to the central conserved region. A further 46 genes that encode similar essential functions map to the left and right terminal regions of the genome and contain 208 substitutions and 14 indels. Because MPXV-SL-V70 and MPXV-ZAI-96 show similar rate of replication and cell-to-cell spread in tissue culture (above), the mutations located in these genes are unlikely to be responsible for the virulence differences noted between the West African and the Congo basin isolates of MPXV.

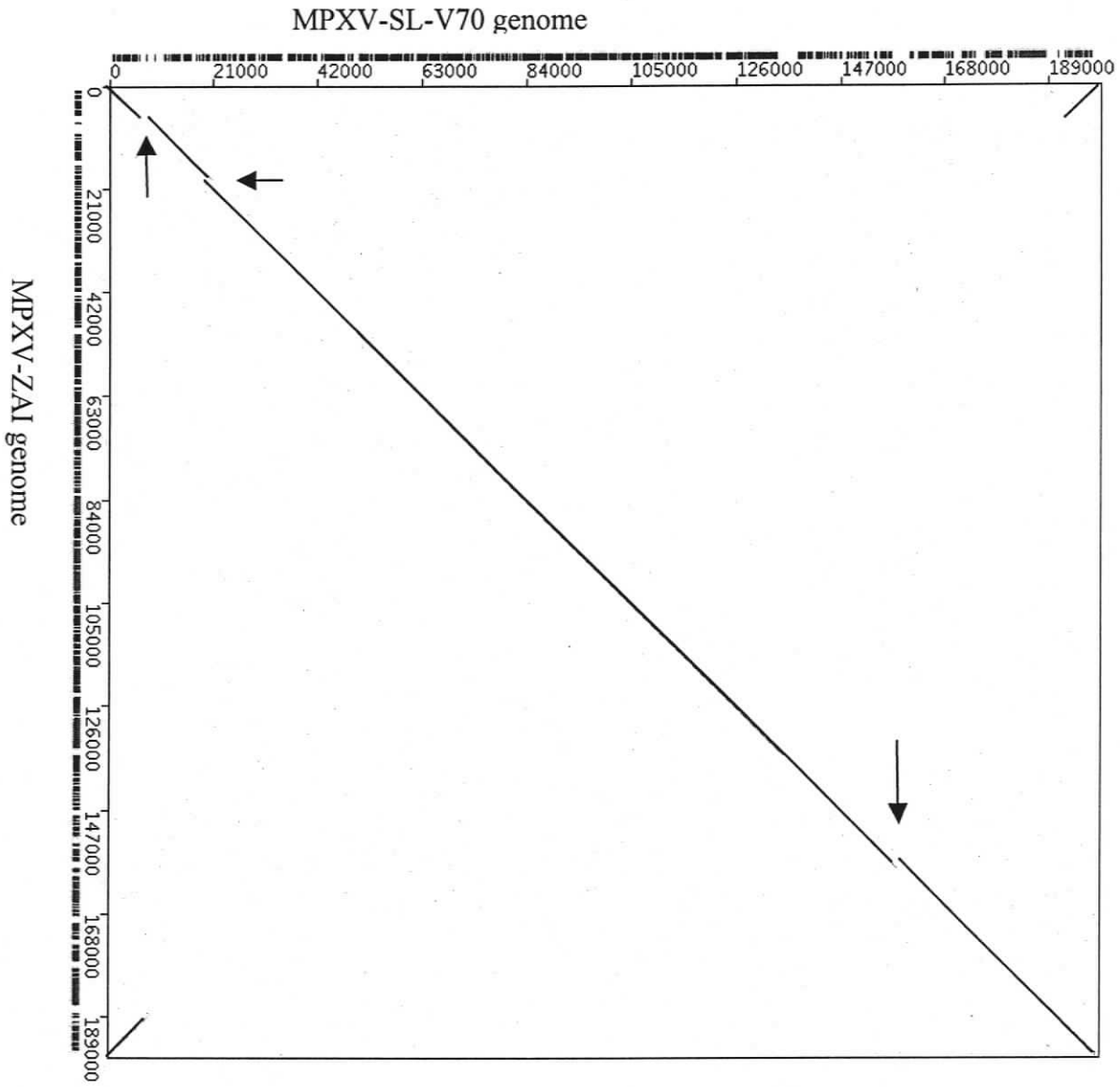


Figure 13 Dotplot of the MPXV-SL-V70 genome and MPXV-ZAI genome. Horizontal axis represents the DNA sequence of the MPXV-SL-V70 genome. Vertical axis represents the DNA sequence of the MPXV-ZAI genome. The arrows show some of the deletions and insertions between the MPXV-SL-V70 and MPXV-ZAI genomes.

Table 7 Fragmented ORFs of MPXV-SL-V70 genome. [1]N-methyl-D-aspartate receptor-associated protein

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

This suggests that the virulence difference noted between West African and Congo basin isolates maps to genes in the terminal regions of the OPV genome that have been shown important for OPV pathogenesis in various animal models, and are predicted to be important for maximizing virus replication, spread, and transmission in the reservoir host species. This group of genes is collectively referred to as the Virulence Ortholog family and MPXV-SL-V70 orthologs contain seven indels and 129 substitutions (Table 8; see footnote 1 for detailed criteria for inclusion in the table). These mutations are responsible for 41 non-conservative and 26 conservative aa changes and changes in the predicted lengths of 7 proteins. The mutational burden in each gene is proportional to its length; although the CPXV-BR-219 ortholog MPXV-SL-V70 171 has 33 mutations over its 1879 aa length, this is 1.76 mutations per 100 aa that is similar to the 1.54 mutations per 100 aa noted for this group of genes as a whole. It is difficult to evaluate the effect of a mutation(s) on protein function without a detailed understanding of the relationship between protein structure and function, but these changes do not suggest any reason to suspect that they significantly contribute to the difference in virulence. Therefore, the remaining 5 genes have been examined in much greater detail since the loss of one of these is more likely to be responsible for the difference in virulence.

MPXV-ZAI-96 D10L, the ortholog of MPXV-SL-V70 013, encodes a host-range function necessary for optimal VACV and ECTV replication in certain tissue culture cell lines, but was not important for ECTV pathogenesis in the A strain of mouse (Chen et al., 1993; Gillard, Spehner, and Drillien, 1985). MPXV-SL-V70 013 has a 4 bp deletion starting 29 bp upstream of the predicted start codon. This mutation brings another

upstream ATG in frame and suggests the possibility of an N-terminal extension to the MPXV-SL-V70 protein that could affect function. This upstream ATG, however, is most likely slightly 5' of the predicted mRNA start site, so I believe it is unlikely that there will be a N-terminal extension for MPXV-SL-V70 as compared to MPXV-ZAI-96 because the translation should start from the normal ATG. This probably eliminates this gene from being a cause of the virulence difference between the isolates.

Table 8 Presence of OPV virulence ortholog family members in MPXV and VARV. [1] Functions thought to be essential for virus replication in standard tissue culture cell lines (*e.g.* functions mainly necessary for transcribing mRNA, replicating the genomic DNA, and assembling infectious virions) are highly conserved in all sequenced OPVs, and map to a central conserved region delineated in VACV-COP by gene F6L (position 38,015; in SL-V70 the ortholog is positioned at 35,132) to A25L (position 138,012; in SL-V70 the ortholog is positioned at 134,611, but is fragmented). For each OPV the remainder of the genome contains a mix of genes, some are specifically tailored to the biology of the individual virus in particular cell types or the reservoir host, and others encode functions essential for all OPVs. These functions are collectively referred to as the Virulence Ortholog family. This set of genes is listed here minus genes with no ascribed function and genes that encode structural components of the virion or nucleic acid metabolism enzymes. [2] Fragment. [3] MOPICE has a single nucleotide deletion leading to a stop codon that terminates the protein 13 aa into the fourth CCP module and 43 aa from the C terminus. [4]Gene is missing

Virulence Ortholog Family[1]	Predicted Function/motif	MPXV				VARV
		SL-V70	COP-58	WRAIR-61	ZAI-96	BSH-75
CPXV-BR-003	CC-chemokine BP	+	+	+	+	+
CPXV-BR-005	TNF BP (Crm B)	+	+	+	+	+
VACV-COP-K3L	EIF-2α homolog	Frag[2]	Frag	Frag	Frag	+
VACV-COP-C11R	Growth factor	+	+	+	+	+
VACV-COP-C10L	IL-1β antagonist	Frag	Frag	Frag	Frag	+
VACV-COP-C4L	IL-1β antagonist-like	+	+	+	+	+
CPXV-BR-023	RING finger/apoptosis	+	+	+	+	+
VARV-BSH-D7L	IL-18 BP	+	+	+	+	+
CPXV-BR-025	Chinese Hamster Ovary Host Range	+	+	+	+	Frag
VACV-COP-C7L	Host range, virulence factor	+	+	+	+	+
VACV-COP-C3L	Inhibitor of complement enzymes	-	-	-	+ (D14L)[3]	+
VACV-COP-N1L	Virulence	+	+	+	+	+
VACV-COP-N2L	α-amanitin sensitivity	+	+	+	+	+
VACV-COP-K1L	Host range	+	+	+	+	Frag
VACV-COP-K2L	Serpin-3 (SPI3)	+	+	+	+	+
VACV-COP-K4L	Phospholipase D-like	+	+	+	+	-[4]
CPXV-BR-045	Putative monoglyceride lipase	+	+	+	+	-
VACV-COP-F1L	Apoptosis inhibitor	+	+	+	+	+
VACV-COP-A38L	CD47-like	+	+	+	+	+
VACV-COP-A42R	Profilin homolog	+	+	+	+	+
VACV-COP-A43R	Membrane protein	+	+	+	+	+
VACV-COP-A44L	Hydroxysteroid dehydrogenase	+	+	+	+	Frag
VACV-COP-A45R	Superoxide dismutase-like	+	+	+	+	+
VACV-COP-A46R	IL-1 signaling inhibitor	+	+	+	+	+
VACV-COP-B7R	Virulence, ER resident	+	+	+	+	-
VACV-COP-B8R	IFN-γ BP	+	+	+	+	+
CPXV-BR-203	Virulence factor	Frag	Frag	Frag	+ (B10R)	-
VACV-COP-B12R	Ser/Thr Kinase	+	+	+	+	Frag
CPXV-BR-207	Serpin-2 (SPI2)	+	+	+	+	+
CPXV-BR-209	IL-1β BP	Frag	Frag	Frag	+ (B14R)	Frag
VACV-COP-B19R	IFN-α/β receptor	+	+	+	+	+
VACV-COP-C12L	Serpin-1 (SPI1)	+	+	+	+	+
CPXV-BR-219	Surface glycoprotein	+	+	+	+	+

MPXV-ZAI-96 D14L encodes MOPICE, an ortholog of VCP (VACV-COP C3L). As compared to VCP, the MOPICE gene has a frame-shifting single nucleotide deletion that generates a premature stop codon and terminates the predicted protein 13 aa into the fourth complement control protein (CCP) module also known as a short consensus repeat (Uvarova and Shchelkunov, 2001). All sequenced Congo basin MPXV isolates (CNG-8, ZAI-V70, ZAI-77, ZAI-96 and ZAI-V79) that were acquired over a 26-year period have an identical MOPICE gene (Uvarova and Shchelkunov, 2001). This gene is completely absent from the genomes of the three West African isolates due to large DNA deletions. MPXV-ZAI-96 B10R encodes a 221 aa protein in the myxoma virus M-T4 virulence factor family characterized by a C-terminal KDEL-like motif in a potential ER-anchoring domain. In poxviruses, this protein is thought to play a role in abrogating apoptosis of infected cells (Barry et al., 1997; Hnatiuk et al., 1999). MPXV-ZAI-96 B10R has orthologs in a variety of poxviruses, but a frameshift mutation removes the C-terminal two-thirds of the predicted protein in all West African isolates. MPXV-ZAI-96 B14R encodes for an IL-1 binding protein that is encoded by most OPVs (Alcami and Smith, 1992; Spriggs et al., 1992), but this ortholog is disrupted by two frame-shifts in all West African isolates. MPXV-ZAI-96 B19R (SPI-I gene) and SPI-I orthologs of several OPVs contain an unusual tandem repeat of CATTATATA immediately upstream of the initiator ATG. The gene of MPXV-SL-V70 169, the ortholog of SPI-I gene, has 7 copies of the repeat compared with 37, 27, 16 identical copies of the sequence in the MPXV-WRAIR-61, MPXV-COP-58, and MPXV-ZAI-96 genomes, respectively. These repeats are positioned between the predicted promoter region and the initiating ATG codon of the SPI-I genes; although there appears to be an in-frame ATG upstream of the MPXV-ZAI-

96 ortholog, our promoter prediction and the primer extension data (Kettle et al., 1997; Kettle et al., 1995) indicate that the mRNA initiates 3 ' of this ATG. Several other OPV genomes (CPXV, VARV, CMLV and VACV-WR) possess a monomer of a similar sequence (CATTATTTA) that may be related to the ancestral sequence of the MPXV-SL-V70 repeats. Although it is not clear what effect the variable lengths of 5' untranslated mRNA, containing these repeats, will have (if any) on the level of SPI-I protein production, it seems unlikely that this gives rise to the difference in virulence because all the MPXV isolates have a variant of this repeat. This SPI-I gene of the West African isolates also has a mutation that causes a Val→Ala change at the P12 position with respect to the reactive center loop; it is not to be expected to affect serpin activity.

Thus, the mutations affecting the West African orthologs of the D14L, B14R and/or B10R genes of the Central African isolate appear to be the best candidates for its increased virulence over the West African isolates.

Since human monkeypox caused by Congo basin isolates of MPXV is almost clinically indistinguishable from smallpox, we further compared genomic sequences of MPXV and VARV to determine if MPXV-ZAI-96 D14L, B10R, and B14R genes are also conserved in VARV. Table 8 shows that while VARV encodes an ortholog to MPXV-ZAI-96 D14L, ORFs corresponding to MPXV-ZAI-96 B10R and B14R orthologs are absent, suggesting that the presence of MOPICE (ORF D14L) in MPXV-ZAI-96, and its absence in three West African isolates makes it the leading candidate responsible for the virulence difference between the West African isolate of MPXV-COP-58 and the Central African

isolate of MPXV-ZAI-V79 (Table 8). Further inspection of Table 8 indicates that 11 genes, which are lacking or truncated in VARV or in the virulent MPXV-ZAI-96 are also probably not essential for OPV virulence in humans (grey high-light). And finally, all of the remaining virulence genes that are conserved in both VARV and MPXV-ZAI-96 may indicate a subgroup of OPV virulence genes important for virulence in humans.

3.2.4 Activity Assay of the MPXV Inhibitor of Complement Enzymes

The inhibitors of complement enzymes, including MOPICE, mimic the biologic activity of complement regulatory proteins (CRPs) that interact with C3b and C4b, thereby inhibiting C3 and C5 convertases (Liszewski et al., 1996). MOPICE consists entirely of a series of four repeating CCP domains; the CCPs are 30-40% identical to the human CRPs including membrane co-factor protein (MCP; CD46), C4-binding protein, Factor H, and decay-accelerating factor (DAF; CD55) and contain the binding sites for C3b and C4b (Herbert et al., 2002). MOPICE, however, is unique among OPV orthologs in that it has a truncated fourth CCP module (Fig. 14A). In spite of this truncation, one preliminary study using a sheep red blood cell hemolysis assay indicates that MOPICE from a Congo basin isolate has complement enzyme inhibitory activity (Smith et al., 2000). To more fully establish the role of MOPICE as a major virulence determinant for the Congo basin MPXV isolates, its inhibitory activity for human complement proteins was characterized by our collaborators in Dr. Mark Buller's lab at the Medical School of Saint Louis University (Fig. 14 B, C and D). Their results indicate that MOPICE, which

lacks most of the fourth CCP, retains complement regulatory activity as detected by Smith (Smith et al., 2000).

The lack of a MOPICE ortholog could make the West African MPXV virions and infected cells more susceptible to antibody and complement mediated lysis; this could diminish virus spread and lead to less severe disease. In addition, the possible enhanced complement cascade activities caused by the lack of MOPICE may have other effects, for example, a stronger antibody response because the breakdown of C3b generates a fragment (C3d) that binds to antigens and enhances their uptake by dendritic and B cells. Consistent with this hypothesis, patients in the USA MPXV 2003 outbreak, as compared to those infected in the Congo basin, had significantly fewer skin lesions and the lesions presented with a unique focal hemorrhagic necrosis possibly due to uncontrolled complement-mediated tissue destruction at the site of infection (Reed et al., 2004). Local increased tissue destruction was also noted in experimental studies in mice with the CPXV mutants lacking a MOPICE ortholog (Kotwal, Miller, and Justus, 1998; Miller et al., 1995; Miller, Shchelkunov, and Kotwal, 1997). Formal proof that MOPICE is a virulent gene will require its deletion in a Congo basin MPXV isolate and further pathogenesis studies in non-human primates.

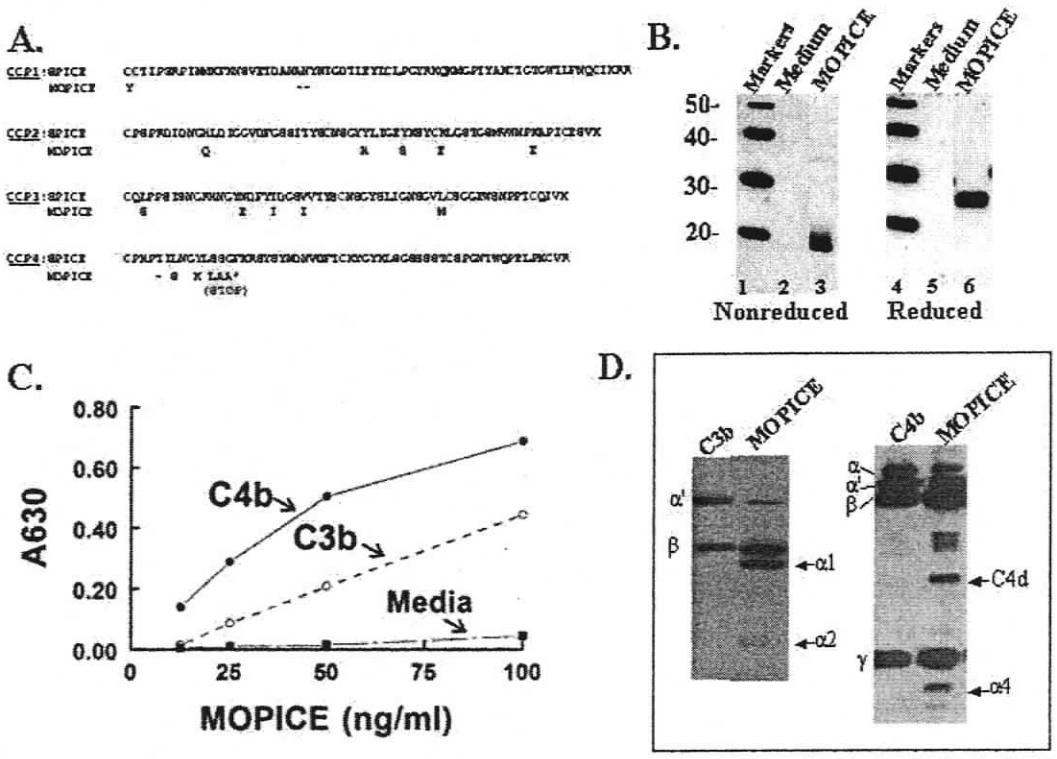


Figure 14 MOPICE structure and function. (A) Amino acid alignment of smallpox inhibitor of complement enzymes (SPICE) and MOPICE without signal peptides illustrating amino acid differences and the premature termination of MOPICE. (B) Western blot of nonreduced and reduced MOPICE. Concentrated CHO supernatants containing MOPICE were electrophoresed in a 10% SDS-PAGE, transferred to nitrocellulose and developed with 1:5000 rabbit anti-VCP antibody. (C) MOPICE binds human C4b and C3b. A representative binding curve is shown. Ligands were coated onto microtiter plates followed by incubations with media or MOPICE. Binding was detected with rabbit anti-VCP antibody (1:5000). MOPICE was quantified in an ELISA (Methods). (D) MOPICE possesses cofactor activity for human C3b and C4b. Chemiluminescent cofactor assays were performed (with or without 10 ng MOPICE), biotinylated human C3b and C4b and human factor I followed by western blot analysis. Arrows denote some of the major cleavage fragments. Controls of MOPICE without factor I did not show cleavage fragments.

Chapter 4 Conclusions

First of all, the phylogenetic analysis in this research show that RPXV-UTR is most closely related to vaccinia virus, although RPXV cannot have evolved directly from any of the sequenced vaccinia strains because RPXV contains a 719 base pair region not previously identified in any vaccinia virus; In addition, three genes, that are present in RPXV-UTR but absent from other VACVs, may explain the enhanced virulence of RPXV over VACV. Each of these genes has features that associate them with poxvirus virulence; some are better characterized than others. For example, knock-out experiments have clearly shown a role for the RING finger protein in ECTV infections of mice (Senkevich, Koonin, and Buller, 1994; Senkevich, Wolffe, and Buller, 1995) and orthologs of the RPXV-UTR-001 protein have been identified as binding host chemokines (Alcami et al., 1998; Lalani et al., 1999; Lalani et al., 1998), but since deletion of the chemokine gene did not attenuate RPXV in mice or rabbits (Martinez-Pomares, Thompson, and Moyer, 1995), it is more likely that one or both of the other genes are responsible for the enhanced RPXV virulence over VACV in rabbits. Although bioinformatics analysis of the RPXV genome cannot substitute for a thorough biochemical characterization of the contribution each of these three genes makes to virus virulence, it may be prudent to ensure that all three of these genes are absent from any VACV strains that are engineered for human vaccines or therapeutics

Secondly, in this research, the collaborator's animal testing indicated that the West African isolate is more virulent in monkey than that of the Central African isolate; further

sequencing and bioinformatics analysis of three MPXV genomes indicate that MPXV-ZAI-96 ORF D14L, which encodes an inhibitor of human complement, is the most promising candidate virulence gene contributing the virulence difference between the West isolates and the Central Africa isolates. These results may explain the lack of case-fatalities in the U.S. 2003 monkeypox outbreak, which was caused by a West African virus.

Finally, it should be noted that there are a large number of minor differences between the genomes that could affect virulence. For example, even single nucleotide changes in poxvirus promoters may significantly alter transcription levels and single amino acid changes in proteins can result in relatively major changes in the protein-protein interactions required for a viral protein to bind a specific host cytokine. In addition, further study of animal model or other experiments is necessary to confirm the hypothesis established from this research.

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Appendix A

Predicted ORFs in the RPXV-UTR Genome. [1] Where possible, proteins have been compared to VACV-COP orthologs. [2] A and B represent the lengths of RPXV-UTR and VACV-COP proteins, respectively, found in BLASTP searches and used to calculate % identity.

RPXV-UTR				VACV-COP[1]		Identity[2]			
ORF	aa	Start	Stop	ORF	aa	A	B	A/B(%)	Putative Function
1	258	2745	1969	C23L	244	239	242	99	Chemokine binding protein
2	184	8260	7706	C16L	181	181	184	98	Unknown
3	91	8874	8599	C15L	91	91	91	100	Unknown
4	192	10263	9685	C14L	82	81	82	99	Unknown
5	357	11498	10425	C12L	353	344	357	96	SPI 1
6	138	11942	12358	C11R	142	134	142	94	EGF-like growth factor
7	331	13506	12511	C10L	331	330	331	100	Similarity to IL-1 Receptor antagonist
8	242	14018	14746	MPXV-ZAI-D5R	242	233	242	96	Ubiquitin ligase activity
9	126	15279	14899	CPXV-BR-024	126	121	126	96	IL-18 BP
10	59	17620	17441	VACV-TT-C10L	59	59	59	100	Unknown
11	634	19704	17800	C9L	634	632	634	100	Ankyrin repeats
12	177	20280	19747	C8L	184	170	184	92	Unknown
13	150	20804	20352	C7L	150	150	150	100	Host range/virulence factor
14	151	21486	21031	C6L	151	151	151	100	Unknown
15	204	22237	21623	C5L	204	204	204	100	Unknown
16	316	23250	22300	C4L	316	316	316	100	IL-1 Receptor antagonist
17	263	24108	23317	C3L	263	260	263	99	Complement control/CD46/EEV
18	512	25713	24175	C2L	512	509	512	99	Kelch repeats
19	224	26457	25783	C1L	224	221	224	99	Unknown
20	117	26797	26444	N1L	117	117	117	100	Virokine/NFkB inhibitor
21	175	27460	26933	N2L	175	170	175	97	Alpha-amanitin sensitivity
22	472	28920	27502	M1L	472	470	472	100	Ankyrin repeats
23	220	29560	28898	M2L	220	220	220	100	NFkB inhibitor
24	284	30549	29695	K1L	284	280	284	99	Ankyrin repeats /NFkB inhibitor
25	369	31880	30771	K2L	369	366	369	99	SPI 3
26	88	32196	31930	K3L	88	88	88	100	IFN resistance/eIF2 alpha-like PKR inhibitor
27	424	33522	32248	K4L	424	423	424	100	Phospholipase D-like
28	149	34478	34927	K7R	149	146	149	98	Unknown
29	227	35678	34995	F1L	226	212	227	93	Apoptosis inhibitor (mitochondria associated)
30	147	36133	35690	F2L	147	144	147	98	dUTPase
31	480	37599	36157	F3L	480	478	480	100	Kelch repeats

32	319	38569	37610	F4L	319	317	319	99	Ribonucleotide reductase (sm. subunit)
33	321	39566	38601	F5L	321	317	321	99	Unknown
34	74	39820	39596	F6L	74	73	74	99	Unknown
35	84	40090	39836	F7L	92	82	92	89	Unknown
36	65	40479	40282	F8L	65	65	65	100	Cytoplasmic protein
37	212	41177	40539	F9L	212	211	212	100	S-S bond formation pathway
38	439	42483	41164	F10L	439	438	439	100	Ser/Thr kinase morphogenesis
39	354	43570	42506	F11L	354	352	354	99	Unknown
40	635	45520	43613	F12L	635	627	635	99	IEV associated
41	372	46680	45562	F13L	372	370	372	99	Phospholipase, EEV
42	73	46919	46698	F14L	73	71	73	97	Unknown
43	158	47669	47193	F15L	158	158	158	100	Unknown
44	231	48371	47676	F16L	231	224	231	97	Unknown
45	101	48434	48739	F17R	101	100	101	99	DNA-binding phosphoprotein
46	479	50175	48736	E1L	479	477	479	100	Poly(A) polymerase (lge. subunit; VP55)
47	737	52385	50172	E2L	737	732	737	99	Unknown
48	190	53084	52512	E3L	190	182	190	96	IFN resistance/PKR inhibitor
49	259	53140	53919	E4L	259	258	259	99	RNA polymerase (RPO30)
50	341	53968	54993	E5R	331	328	331	99	Virosome component
51	567	55130	56833	E6R	567	565	567	100	Unknown
52	166	56917	57417	E7R	166	162	166	98	Soluble/Myristyl EEV
53	273	57542	58363	E8R	273	271	273	99	ER-localized MP
54	1006	61389	58369	E9L	1006	1005	1006	100	DNA polymerase
55	95	61421	61708	E10R	95	94	95	99	S-S formation pathway
56	129	62092	61703	E11L	129	129	129	100	Virion core protein
57	666	64079	62079	O1L	666	663	666	100	Unknown
58	108	64453	64127	O2L	108	108	108	100	Glutaredoxin 1
59	312	65537	64599	I1L	312	312	312	100	DNA-binding protein
60	73	65765	65544	I2L	73	73	73	100	Unknown
61	269	66575	65766	I3L	269	266	269	99	DNA-binding phosphoprotein
62	771	68973	66658	I4L	771	771	771	100	Ribonucleotide reductase (lge. subunit)
63	79	69239	69000	I5L	79	79	79	100	IMV protein VP13
64	382	70406	69258	I6L	382	382	382	100	Telomere binding protein
65	423	71670	70399	I7L	423	420	423	99	Virion core protease
66	676	71676	73706	I8R	676	673	676	100	RNA helicase/NPH-II
67	591	75485	73710	G1L	591	589	591	100	Predicted metallo-protease
68	111	75817	75482	G3L	111	111	111	100	Unknown
69	220	75811	76473	G2R	220	219	220	100	VLTF
70	124	76817	76443	G4L	124	124	124	100	Glutaredoxin 2
71	434	76820	78124	G5R	434	428	434	99	Unknown
72	63	78132	78323	G5.5R	63	63	63	100	RNA polymerase (RPO7)
73	165	78325	78822	G6R	165	164	165	99	Unknown
74	371	79902	78787	G7L	371	370	371	100	Virion assembly protein
75	260	79933	80715	G8R	260	260	260	100	VLTF-1
76	340	80735	81757	G9R	340	339	340	100	Myristylated protein
77	250	81758	82510	L1R	250	249	250	100	Myrisylated MP IMV
78	87	82542	82805	L2R	87	87	87	100	Unknown

79	350	83847	82795	L3L	350	345	350	99	Unknown
80	251	83872	84627	L4R	251	251	251	100	Core package/transcription
81	128	84637	85023	L5R	128	127	128	99	Putative MP
82	153	84980	85441	J1R	153	152	153	99	Virion morphogenesis
83	177	85457	85990	J2R	177	177	177	100	Thymidine kinase
84	333	86056	87057	J3R	333	326	333	98	Poly(A) polymerase (sm. subunit; VP39)
85	185	86972	87529	J4R	185	185	185	100	RNA polymerase (RPO22)
86	133	88040	87639	J5L	133	131	133	98	Unknown MP
87	1286	88146	92006	J6R	1286	1283	1286	100	RNA polymerase (RPO147)
88	171	92518	92003	H1L	171	169	171	99	Tyr/Ser phosphatase
89	189	92532	93101	H2R	189	188	189	99	Entry/cell-cell fusion
90	324	94078	93104	H3L	324	320	324	99	IMV heparin binding surface protein
91	795	96466	94079	H4L	795	794	795	100	RAP94 (RNA pol assoc protein)
92	203	96652	97263	H5R	203	202	203	100	VLTF-4
93	314	97264	98208	H6R	314	314	314	100	Topoisomerase type I
94	146	98245	98685	H7R	146	143	146	98	Unknown
95	844	98729	101263	D1R	844	843	844	100	Capping enzyme (lge. subunit)
96	146	101662	101222	D2L	146	146	146	100	Virion core
97	237	101655	102368	D3R	237	234	237	99	Virion core
98	218	102368	103024	D4R	218	218	218	100	Uracil-DNA glycosylase
99	785	103056	105413	D5R	785	779	785	99	NTPase, DNA replication
100	637	105454	107367	D6R	637	635	637	100	Morphogenesis, VETF-s
101	161	107394	107879	D7R	161	160	161	99	RNA polymerase (RPO18)
102	304	108756	107842	D8L	304	300	304	99	Carbonic anhydrase/virion
103	213	108798	109439	D9R	213	212	213	100	mutT motif/NTP-PPH
104	248	109436	110182	D10R	248	247	248	100	mutT motif/NPH-PPH/down regulator
105	631	112078	110183	D11L	631	628	631	100	NPH-I, virion
106	287	112976	112113	D12L	287	286	287	100	Capping enzyme (sm. subunit)
107	551	114662	113007	D13L	551	549	551	100	Rifampicin resistance
108	150	115138	114686	A1L	150	150	150	100	VLTF-2
109	224	115833	115159	A2L	224	224	224	100	VLTF-3
110	76	116060	115830	A2.5L	74	74	74	100	Thioredoxin-like
111	644	118009	116075	A3L	644	643	644	100	P4b precursor
112	283	118913	118062	A4L	281	276	283	98	Core protein
113	164	118951	119445	A5R	164	164	164	100	RNA polymerase (RPO19)
114	372	120560	119442	A6L	372	370	372	99	Unknown
115	710	122716	120584	A7L	710	709	710	100	VETF (lge. subunit
116	288	122770	123636	A8R	288	288	288	100	VITF-3 (sm. subunit)
117	99	123928	123629	A9L	99	98	99	99	Membrane protein
118	892	126607	123929	A10L	891	885	892	99	P4a precursor
119	318	126622	127578	A11R	318	316	318	99	Viral membrane formation
120	192	128158	127580	A12L	192	190	192	99	Structural protein
121	70	128394	128182	A13L	70	69	69	100	Virion maturation
122	90	128774	128502	A14L	90	90	90	100	IMV PO4 MP
123	53	128791	128952	A14.5L	53	53	53	100	IMV-MP/Virulence factor
124	94	128942	129226	A15L	94	94	94	100	Unknown
125	378	130346	129210	A16L	378	378	378	100	Soluble/Myristylated
126	203	130960	130349	A17L	203	203	203	100	IMV MP PO4

127	493	130975	132456	A18R	493	488	493	99	DNA Helicase, transcription
128	77	132670	132437	A19L	77	77	77	100	Unknown
129	117	133024	132671	A21L	117	115	117	98	Unknown
130	426	133023	134303	A20R	426	423	426	99	DNA processivity factor
131	187	134233	134796	A22R	176	175	176	99	Resolvase
132	382	134816	135964	A23R	382	382	382	100	VITF-3 (lge. subunit)
133	1164	135961	139455	A24R	1164	1161	1164	100	RNA polymerase (RPO 132)
134	500	144773	143271	A26L	322	196	197	99	p4c virion occlusion protein
135	110	145155	144823	A27L	110	108	110	98	Fusion protein
136	146	145596	145156	A28L	146	145	146	99	IMV MP/Virus entry
137	305	146514	145597	A29L	305	304	305	100	RNA polymerase (RPO35)
138	77	146710	146477	A30L	77	77	77	100	Virion morphogenesis
139	124	146870	147244	A31R	124	123	124	99	Unknown
140	300	148113	147211	A32L	300	299	300	100	ATPase/DNA packaging protein
141	185	148141	148698	A33R	185	185	185	100	EEV Glycoprotein
142	168	148722	149228	A34R	168	166	168	99	EEV Glycoprotein
143	177	149272	149805	A35R	176	176	177	99	Unknown
144	221	149872	150537	A36R	221	221	221	100	IEV-specific
145	263	150601	151392	A37R	263	257	262	98	Unknown
146	277	152506	151673	A38L	277	274	277	99	CD47-like
147	401	152523	153728	A39R	403	395	403	98	Semaphorin
148	159	153754	154233	A40R	168	134	137	98	Lectin like
149	219	154990	154331	A41L	219	215	219	98	Secreted/Virulence
150	133	155155	155556	A42R	133	133	133	100	Profilin-like
151	194	155594	156178	A43R	194	194	194	100	Membrane glycoprotein-class I
152	78	156186	156422	VACV-WR-169	78	78	78	100	Unknown
153	346	157558	156518	A44L	346	342	346	99	Hydroxysteroid dehydrogenase
154	125	157605	157982	A45R	125	123	125	98	Superoxide dismutase-like
155	240	157972	158694	A46R	214	185	185	100	IL-1 Signaling inhibitor
156	244	159516	158782	A47L	244	243	244	100	Unknown
157	204	159615	160229	A48R	204	203	204	100	Thymidylate kinase
158	162	160277	160765	A49R	162	160	162	99	Unknown
159	552	160798	162456	A50R	552	550	552	100	DNA ligase
160	190	163580	164152	A52R	190	190	190	100	TLR & IL-1 signalling receptor
161	102	164518	164826	A53R	103	68	74	92	TNF-alpha receptor-like
162	564	165322	167016	A55R	564	554	564	98	Kelch-like
163	206	167383	168003	A56R	315	203	209	97	Hemaglutinin
164	151	168148	168603	A57R	151	146	151	97	Guanylate kinase
165	300	168754	169656	B1R	300	299	300	100	Ser/Thr Kinase
166	558	171473	173149	B4R	558	553	558	99	Ankyrin repeats
167	317	173253	174206	B5R	317	317	317	100	Complement control/CD46/EEV
168	173	174302	174823	B6R	173	168	173	97	Unknown
169	182	174861	175409	B7R	182	181	182	99	Virulence , ER resident
170	272	175464	176282	B8R	272	267	272	98	IFN-gamma receptor-like
171	77	176526	176293	unannotated	58	48	48	100	Unknown
172	166	176564	177064	B10R	166	166	166	100	Kelch-like
173	72	177136	177354	B11R	88	68	71	96	Unknown
174	283	177421	178272	B12R	283	280	283	99	Ser/Thr kinase
175	345	178372	179409	VACV-WR-195	222	210	219	96	Serpin 2

176	149	179486	179935	B15R	149	148	149	99	Unknown
177	340	182099	181077	B17L	340	337	340	99	Unknown
178	574	182236	183960	B18R	574	568	574	99	Ankyriin repeats
179	351	184032	185087	B19R	353	347	353	98	IFN-alpha/beta receptor
180	791	185181	187556	B20R	127	123	123	100	Ankyrin repeats
181	82	187799	188047	C14L	82	81	82	99	Unknown
182	91	188858	189133	C15L	91	91	91	100	Unknown
183	184	189472	190026	C16L	181	181	184	98	Unknown
184	258	194987	195763	C23L	244	239	242	99	Chemokine binding protein

Appendix B

Predicted ORFs in the MPXV-SL-V70 Genome. [1] DNA encoding remnants of CPXV-BR-001, -002, -063, 174, -216, -228, -229, and VACV-COPC-C15L was present, but the residual coding sequences were not annotated; [2]ZAI-96 genome was reannotated as described in the Methods section. This process removes ORFs that are vestiges of conserved ORFs present in other poxviruses or small predicted ORFs on the non-coding strand; [3]Length, number of aa in ORF; [4]Start, first nucleotide of start codon; [5]Stop, last nucleotide of stop codon; [6]Orthologus ORF in the VACV-COP, unless otherwise indicated (MOS, ECTV-MOS; BR, CPXV-Brighton Red; BSH, VARV-BSH; TIA, VACV-TIA; MVA, VACV-MVA, and ZAI, ZAI-96); [7]An ortholog not present in the corresponding region of ZAI-96; [8]An ortholog is not present in SL-V70; [9] SL-V70 ORF 163 is the single gene predicted in the SL-V70 isolate, and a number of other OPVs that is not annotated ZAI-96. Orthologs of this predicted protein range in size from 72-106 due to a highly variable N-terminal region that contains different lengths of an Asp-Thr repeat. A single insertion/deletion (indel), approximately one third into the ORF, induces a frameshift that results in an early termination codon in an otherwise complete ZAI-96 gene. Since a promoter has not been characterized for this predicted ORF, it is not possible to predict what polypeptides are likely to be made by the viruses of these two groups. Database searches with these predicted proteins failed to yield any significant matches to non-OPV proteins. Abbreviations: secP, secreted protein; BP, binding protein; CHO, Chinese hamster ovary; P, protein; MG, monoglyceride lipase; R., ribonucleotide; inter, interaction; MP, membrane protein; IEV, intracellular enveloped virion; EEV, extracellular enveloped virion; PP, phosphoprotein; pol, polymerase; PKR, dsRNA-dependent protein kinase; OAS, 2′–5′ oligoadenylate synthetase;VITF, viral intermediate transcription factor; myristyl P, myristylated protein; PW, pathway; IMV, intracellular mature virion; morphogen., morphogenesis; N triphosphat, nucleotide triphosphatase; CP, cysteine proteinase; VLTF, viral late transcription factor; topo, topoisomerase; VETF, viral early transcription factor; attach., attachment; rif resist, rifampicin resistance; IF, inclusion factor; TM, transmembrane; DH, dehydrogenase; HA, hemagglutinin. Predictions: secreted proteins by SignalP V1.1; membrane proteins by TMpred.

MPXV-SL-V70[1]				MPXV-ZAI-96 Ortholog[2]				Predicted Function/Motif
Name	Length[3]	Start[4]	Stop[5]	Name	Leng	Identical	Identity (%)	
1	246	1510	770	J1L	246	246	100.0	SecP/CC-Chemokine BP (C23L/B29R)[6]
2	349	2686	1637	J2L	348	345	98.9	SecP/TNF BP (crmB) (BR005/226)
3	590	4548	2776	J3L	587	581	99.2	Ankyrin/unknown

								(BR-006/225)
4	437	6008	4695	D1L	437	432	98.9	Ankyrin/unknown (BR-017)
5[7]	176	6653	6123	-	-	-	-	Unknown (BR-018)
6[7]	153	7863	7402	-	-	-	-	Unknown (C16L/B22R)
7	142	9076	9504	D3R	142	142	100.0	Growth factor (C11R)
8	242	11081	11809	D5R	242	240	99.2	RING finger/apoptosis (MOS-012)
9	126	12392	12012	D6L	126	126	100.0	SecP/IL-18 BP (BSH-D7L)
10	660	14434	i2452	D7L	660	650	98.5	CHO Host range (BSH-D8L)
11	64	14764	14570	D8L	64	63	98.4	Retroviral pseudoprotease (BR-026)
12	630	16803	14911	D9L	630	626	99.4	Ankyrin (C9L)
13	167	17964	17461	D10L	150	147	98.0	Host range (C7L)
14	159	18617	18138	D11L	153	151	99.3	Unknown (C6L)
15	206	19381	18761	D12L	206	204	99.0	Unknown (C5L)
16	316	20376	19426	D13L	315	312	98.7	IL-1 receptor antagonist (C4L)
-	-	-	-	D14L[8]	216	-	-	Inhibitor of complement enzymes (C3L)
17	214	21561	20917	D19L	214	213	99.5	Unknown (C1L)
18	117	21960	21607	P1L	117	117	100.0	Cytoplasmic P/virulence (N1L)
19	177	22620	22087	P2L	177	176	99.4	α-amanitin sensitivity (N2L)
20	446	23988	22648	O1L	442	439	99.3	Ankyrin/unknown (M1L)
21	220	24720	24058	O2L	220	218	99.1	Unknown (M2L)
22	284	25679	24825	C1L	284	283	99.7	Ankyrin/host range (K1L)
23	374	27422	26298	C2L	375	373	99.5	Serpin (SPI-3)/unknown (K2L)
24	424	29041	27767	C4L	424	423	99.8	Phospholipase D-like/unknown (K4L)
25	276	29899	29069	C5L	276	274	99.3	MG lipase-like/unknown (BR045)
26	149	30035	30484	C6R	149	146	98.0	Unknown (K7R)
27	219	31206	30547	C7L	219	216	98.6	Apoptosis inhibitor (F1L)
28	151	31673	31218	C8L	151	151	100.0	dUTPase (F2L)
29	492	33148	31670	C9L	487	477	98.4	Kelch-like/unknown (F3L)
30	319	34118	33159	C10L	319	319	100.0	R.Reductase-small (F4L)
31	343	35180	34149	C11L	343	339	98.8	Major membrane protein (F5L)
32	73	35358	35137	C12L	73	73	100.0	Unknown (F6L)
33	74	35598	35374	C13L	74	74	100.0	Unknown (F7L)
34	64	35944	35750	C14L	64	64	100.0	Proline rich P/unknown (F8L)
35	212	36639	36001	C15L	212	212	100.0	Putative MP/unknown (F9L)
36	439	37945	36626	C16L	439	438	99.8	Ser/Thr kinase/morphogen (F10L)
37	354	39032	37968	C17L	354	353	99.7	Unknown (F11L)
38	635	40983	39076	C18L	635	630	99.2	IEV, actin tail, microtubule inter. (F12L)
39	372	42144	41026	C19L	372	370	99.5	Phospholipase/EEV (F13L)
40	73	42383	42162	C20L	73	73	100.0	Unknown (F14L)
41	158	43131	42655	C21L	158	158	100.0	Unknown (F15L)
42	231	43833	43138	C22L	231	231	100.0	MP/unknown (F16L)

43	101	43896	44201	C23R	101	101	100.0	IMV, DNA bound PP (F17R)
44	479	45637	44198	F1L	479	477	99.6	Poly(A) pol large (E1L)
45	737	47847	45634	F2L	737	736	99.9	Unknown (E2L)
46	153	48432	47971	F3L	153	153	100	PKR/OAS inhibitor (E3L)
47	259	49377	48598	F4L	259	257	99.2	RNA pol (RPO30) VITF-01 (E4L)
48	567	50150	51853	F5R	567	565	99.7	Unknown (E6R)
49	166	51935	52435	F6R	166	166	100.0	myristyl MP/EEV (E7R)
50	273	52539	53360	F7R	273	272	99.6	ER-localized MP/unknown (E8R)
51	1006	56387	53367	F8L	1006	1002	99.6	DNA pol (E9L)
52	95	56419	56706	F9R	95	93	97.9	IMV, -S-S-bond PW (E10R)
53	129	57090	56701	F10L	129	129	100.0	IMV, core (E11L)
54	665	59074	57077	Q1L	665	663	99.7	MP/unknown (O1L)
55	108	59447	59121	Q2L	108	108	100.0	Glutaredoxin/unknown (O2L)
56	312	60532	59594	I1L	312	312	100.0	IMV, core, morphogen (I1L)
57	73	60760	60539	I2L	73	73	100.0	MP/unknown (I2L)
58	269	61570	60761	I3L	269	268	99.6	DNA-binding PP (I3L)
59	771	63967	61652	I4L	771	764	99.1	R.Reductase-large (I4L)
60	79	64235	63996	I5L	79	79	100.0	MP/IMV (I5L)
61	382	65402	64254	I6L	382	381	99.7	Telomere BP (I6L)
62	423	66666	65395	I7L	423	423	100.0	IMV, core, CP (I7L)
63	676	66672	68702	I8R	676	675	99.9	RNA helicase, NPH-II (I8R)
64	591	70481	68706	G1L	591	587	99.5	Metalloprotease (G1L)
65	220	70807	71469	G3R	220	220	100.0	VLTF (G2R)
66	111	70813	70478	G2L	111	111	100.0	SecP/unknown (G3L)
67	124	71813	71439	G4L	124	124	100.0	IMV, –S-S- bond PW (G4L)
68	434	71816	73120	G5R	434	431	99.3	Unknown (G5R)
69	63	73129	73320	G6R	63	63	100.0	RNA pol (RPO7) (G5.5R)
70	165	73320	73817	G7R	165	162	98.2	Unknown (G6R)
71	371	74897	73782	G8L	371	370	99.7	IMV, core, matrix (G7L)
72	260	74928	75710	G9R	260	260	100.0	VLTF-1 (G8R)
73	340	75730	76752	G10R	340	340	100.0	Myristyl MP/unknown (G9R)
74	250	76753	77505	M1R	250	250	100.0	myristyl MP/IMV (L1R)
75	92	77537	77815	M2R	92	91	98.9	MP/unknown (L2R)
76	344	78825	77791	M3L	344	343	99.7	Unknown (L3L)
77	251	78850	79605	M4R	251	251	100.0	IMV, core, ssDNA binding (L4R)
78	128	79615	80001	M5R	128	128	100.0	MP/unknown (L5R)
79	152	79958	80416	L1R	152	151	99.3	MP/IMV, morphogen (J1R)
80	177	80436	80969	L2R	177	175	98.9	Thymidine kinase (J2R)
81	333	81035	82036	L3R	333	331	99.4	Poly(A) poly-small (VP39) (J3R)
82	185	81951	82508	L4R	185	185	100.0	RNA pol (RPO22) (J4R)
83	133	82971	82570	L5L	133	133	100.0	MP/unknown (J5L)
84	1286	83078	86938	L6R	1286	1278	99.4	RNA pol (RPO147) (J6R)
85	171	87450	86935	H1L	171	171	100.0	Tyr/Ser phosphatase/unknown (H1L)

86	189	87464	88033	H2R	189	188	99.5	MP/unknown (H2R)
87	324	89011	88037	H3L	324	322	99.4	MP/IMV (H3L)
88	795	91399	89012	H4L	795	791	99.5	RNA pol assoc P 94 (H4L)
89	210	91584	92216	H5R	213	208	97.7	VLTF-4 (H5R)
90	314	92217	93161	H6R	314	313	99.7	DNA topo type I (H6R)
91	144	93199	93633	H7R	146	143	99.3	MP/unknown (H7R)
92	845	93677	96214	E1R	845	843	99.8	Capping enzyme-large (D1R)
93	233	96606	97307	E3R	233	233	100.0	IMV, core (D3R)
94	146	96613	96173	E2L	146	146	100.0	IMV, core (D2L)
95	218	97307	97963	E4R	218	218	100.0	Uracil-DNA glycosylase (D4R)
96	785	97995	100352	E5R	785	784	99.9	N. triphosphat. /DNA replication (D5R)
97	637	100392	102305	E6R	637	635	99.7	VETF-small (D6R)
98	161	102332	102817	E7R	161	161	100.0	RNA pol (RPO18) (D7R)
99	304	103694	102780	E8L	304	302	99.3	MP/IMV, attach (D8L)
100	213	103736	104377	E9R	213	213	100.0	MutT-like/unknown (D9R)
101	248	104374	105120	E10R	248	248	100.0	MutT-like/unknown (D10R)
102	631	107016	105121	E11L	631	627	99.4	NPH-I/IMV (D11L)
103	287	107914	107051	E12L	287	287	100.0	Capping enzyme-small (D12L)
104	551	109600	107945	E13L	551	548	99.5	IMV, morphogen, rif resist (D13L)
105	150	110076	109624	A1L	150	150	100.0	VLTF-2 (A1L)
106	224	110771	110097	A2L	224	223	99.6	VLTF-3 (A2L)
107	77	111001	110768	A3L	77	77	100.0	Thioredoxin/-S-S-bond PW (A2.5L)
108	644	112950	111016	A4L	644	644	100.0	IMV, core, precursor of p4b (A3L)
109	281	113848	113003	A5L	281	278	98.9	IMV, matrix, morphogen (A4L)
110	161	113886	114371	A6R	161	161	100.0	RNA pol (RPO19) (A5R)
111	372	115486	114368	A7L	372	372	100.0	Unknown (A6L)
112	710	117642	115510	A8L	710	709	99.9	VETF-large (A7L)
113	292	117696	118574	A9R	292	290	99.3	VITF-3-S (A8R)
114	112	118893	118555	A10L	100	99	88.4	MP/IMV, morphogen(A9L)
115	891	121569	118894	A11L	891	888	99.7	IMV, core, precursor of p4a (A10L)
116	318	121584	122540	A12R	318	318	100.0	MP/unknown (A11R)
117	190	123114	122542	A13L	190	190	100.0	IMV, core (A12L)
118	70	123350	123138	A14L	70	70	100.0	MP/IMV (A13L)
119	90	123728	123456	A15L	90	90	100.0	MP/IMV, morphogen (A14L)
120	53	123906	123745	A15.5L	53	53	100.0	MP/IMV, virulence (A14.5L)
121	94	124180	123896	A16L	94	94	100.0	Unknown (A15L)
122	377	125297	124164	A17L	377	374	99.2	Myristyl P/unknown (A16L)
123	204	125914	125300	A18L	204	203	99.5	MP/IMV, morphogen (A17L)
124	492	125929	127407	A19R	492	489	99.4	IMV, core, DNA helicase (A18R)
125	77	127621	127388	A20L	77	76	98.7	Unknown (A19L)
126	426	127968	129248	A22R	426	425	99.8	DNA pol processivity (A20R)
127	115	127969	127622	A21L	115	114	99.1	SecP/unknown (A21L)

128	187	129178	129741	A23R	187	187	100.0	Holiday junction resolvase (A22R)
129	382	129761	130909	A24R	382	381	99.7	VITF-3L (A23R)
130	1164	130906	134400	A25R	1164	1160	99.7	RNA pol (RPO132) (A24R)
131	506	139626	138106	A28L	520	501	96.4	MP/IMV, P4c IF (BSH A30L)
132	110	140009	139677	A29L	110	108	98.2	MP/IMV (A27L)
133	146	140450	140010	A30L	146	146	100.0	SecP TM/unknown (A28L)
134	305	141368	140451	A31L	305	301	98.7	RNA pol (RPO35) (A29L)
135	78	141567	141331	A32L	77	76	97.4	IMV, matrix, morphogen (A30L)
136	142	141727	142155	A33R	142	140	98.6	Unknown (A31R)
137	42	141728	141600	A32.5L	42	42	100.0	Unknown (A30.5L)
138	300	143024	142122	A34L	300	298	99.3	ATPase/DNA packaging (A32L)
139	181	143052	143597	A35R	181	180	99.5	MP/CEV, EEV (A33R)
140	168	143602	144108	A36R	168	167	99.4	MP/CEV, EEV (A34R)
141	176	144152	144682	A37R	176	175	99.4	Unknown (A35R)
142	228	144728	145414	A38R	212	208	98.1	MP/IEV (A36R)
143	268	145466	146272	A39R	268	264	98.5	Unknown (A37R)
144	277	147357	146524	A40L	277	275	99.3	MP, CD47-like/unknown (A38L)
145	221	148758	148093	A41L	221	219	99.1	SecP/virulence (A41L)
146	133	148961	149362	A42R	133	133	100.0	Profilin-like (A42R)
147	196	149400	149990	A43R	197	193	98.5	MP/unknown (A43R)
148	74	150010	150234	A44R	74	73	98.7	Unknown (MVA-156R)
149	346	151370	150330	A45L	346	345	99.7	Hydroxysteroid DH (A44L)
150	125	151417	151794	A46R	125	125	100.0	Superoxide dismutase-like (A45R)
151	240	151784	152506	A47R	240	239	99.6	Inhibits NF-κB activation (A46R)
152	204	153459	154073	A49R	204	203	99.5	Thymidylate kinase (A48R)
153	559	154642	156321	A50R	554	554	100.0	DNA ligase (A50R)
154	334	156362	157366	A51R	334	331	99.1	Unknown (A51R)
155	313	160850	161791	B2R	313	310	99.0	MP/CEV, EEV, HA (A56R)
156	303	162553	163464	B3R	299	299	100.0	Ser/Thr kinase/unknown (B1R)
157	505	163520	165037	B4R	503	500	99.4	Unknown (B2R/B3R)
158	564	165226	166920	B5R	561	559	99.1	Ankyrin/unknown (B4R)
159	317	167024	167977	B6R	317	316	99.7	MP/CEV, EEV (B5R)
160	176	168049	168579	B7R	176	174	98.9	MP/unknown (B6R)
161	182	168617	169165	B8R	182	180	98.9	ER P/virulence (B7R)
162	267	169220	170023	B9R	267	267	100.0	SecP/IFN-γ BP (B8R)
-	-	-	-	B10R[8]	221	-		Virulence factor (BR-203)
163[7,9]	98	171559	171855	-	-	-	-	Unknown (COP-11R)
164	282	171921	172769	B11R	282	280	99.3	Ser/Thr kinase/unknown (B12R)
165	344	172869	173903	B12R	344	343	99.7	Serpin (SPI-2)/apoptosis (BR-207)
166	149	174030	174479	B13R	149	146	98.0	MP/unknown (B15R)
-	-	-	-	B14R[8]	326	-	-	IL-1β BP (BR-209)
167	352	176361	177419	B16R	352	351	99.7	IFN-α/β BP (B19R)
168	787	177488	179851	B17R	793	781	98.5	Ankyrin/unknown (BSH-B18R)

169	397	180861	182054	B19R	357	352	98.6	Serpin (SPI-1)/unknown (C12L)
170	190	182226	182798	B20R	190	187	98.4	MP/unknown (BR-218)
171	1880	183055	188697	B21R	1879	1861	99.0	MP/unknown (BSH B22R)
172	153	190894	191355	N1R	153	152	99.4	Unknown (B22R)
173	176	192104	192634	N3R	176	174	98.9	Unknown (BR-018)
174	437	192749	194062	N4R/D1L	437	432	98.9	Ankyrin/unknown (BR-017)
175	590	194209	195981	J1R	587	581	99.2	Ankyrin/unknown (BR-006/225)
176	349	196071	197120	J2R	348	345	98.9	SecP/TNF BP (crmB) (BR-005/ 226)
177	246	197247	197987	J3R	246	246	100.0	SecP/CC chemokine BP (C23L/B29R)

Appendix C Publications from This Thesis

Chen, N., Li, G., Liszewski, M. K., Atkinson, J. P., Jahrling, P. B., Feng, Z., Schriewer, J., Buck, C., Wang, C., Lefkowitz, E. J., Esposito, J. J., Harms, T., Damon, I. K., Roper, R. L., Upton, C., and Buller, R. M. (2005). Virulence differences between monkeypox virus isolates from West Africa and the Congo basin. *Virology* 340(1), 46-63.

Li, G., Chen, N., Roper, R. L., Feng, Z., Hunter, A., Danila, M., Lefkowitz, E. J., Buller, R. M., and Upton, C. (2005). Complete coding sequences of the rabbitpox virus genome. *J Gen Virol* 86(Pt 11), 2969-77.