Molecular and Biological Characterization of a Tanapox Virus TNF-Binding Protein and the Complete Nucleotide Sequence of the PstI-J Genomic Fragment

Mini Paulose-Murphy
Western Michigan University

Follow this and additional works at: https://scholarworks.wmich.edu/dissertations
Part of the Biology Commons

Recommended Citation
Paulose-Murphy, Mini, "Molecular and Biological Characterization of a Tanapox Virus TNF-Binding Protein and the Complete Nucleotide Sequence of the PstI-J Genomic Fragment" (2000). Dissertations. 3447.
https://scholarworks.wmich.edu/dissertations/3447
MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF A TANAPOX VIRUS TNF-BINDING PROTEIN AND THE COMPLETE NUCLEOTIDE SEQUENCE OF THE PstI-J GENOMIC FRAGMENT

by

Mini Paulose-Murphy

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
April 2000
Copyright by
Mini Paulose-Murphy
2000
I would like to begin by acknowledging my mentor Dr. Karim Essani for his endless patience, guidance, and insight. I am indebted to him for my training and development as a scientist. I would like to thank the members of my committee, Drs. Robert Eisenberg, Bruce Bejcek, Tony Manning, and Ronald Shebuski for taking the time to review this dissertation and for their expertise and assistance throughout the duration of this work. I would also like to extend my sincere thanks to my colleagues Bina Garimella, Celene Jackson, Takeshi Shimamura, Julie Stahlhut, Scott Haller, Vivian Locke and Steve Conrad for their friendship. Also, a special thank you to Dr. Robert Eversole for his invaluable technical and electron microscopy assistance and Dr. Dewayne Shoemaker and Santiago Navarro for their expertise in DNA sequencing.

I would like to lovingly acknowledge my husband, Kelly Thomas Murphy for his friendship, devotion, and constant support. I would like to wholeheartedly thank my sister Ryne, my brother Mathew, and my best friend Janine for their encouragement and support. And lastly but never least, to my parents, words cannot express my gratitude for their unconditional love and guidance. This work is dedicated to my parents.

Mini Paulose-Murphy
MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF A TANAPOX VIRUS TNF-BINDING PROTEIN AND THE COMPLETE NUCLEOTIDE SEQUENCE OF THE PstI-J GENOMIC FRAGMENT

Mini Paulose-Murphy, Ph.D.
Western Michigan University, 2000

Most of the poxvirus encoded virulence factors have been identified as proteins that are secreted from infected host cells. Some of these secretory proteins impede host immune defenses. We have previously demonstrated that tanapox virus (TPV) infected cells secrete a 38 kDa glycopeptide that binds to human (h) interferon-γ, h-interleukin (IL)-2, and hIL-5. We now show an additional activity in the supernatant from TPV infected cells that down-regulates the expression of tumor necrosis factor-α (TNF-α) induced cell adhesion molecule gene expression. This activity was not detected in mock infected cells. Enzyme linked immunosorbent assays (ELISA) on primary human endothelial cells, show the induction of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) following TNF-α or IL-1β treatment, as expected. Supernatant from TPV infected cells significantly decreased the TNF-α induced activation of the nuclear transcription factor-κB (NF-κB) and transcriptional activation of the E-selectin, VCAM-1, and ICAM-1 genes. Based on TNF-α affinity chromatography, this activity appears to be associated with a 38 kDa protein. This 38 kDa protein was purified and 25 amino acid residues sequenced from the N-terminus. Multiple oligonucleotide
probes were designed from this protein sequence to identify the gene encoding the 38 kDa protein. The 5.1 kbp TPV PstI-J genomic fragment was isolated and both strands completely sequenced. Analyses of the TPV PstI-J fragment revealed 7 potential open reading frames with significant homology to other poxviruses including vaccinia virus and Yaba virus. Careful analyses of these open reading frames did not reveal any homology to the N-terminal 25 amino acid sequence of the 38 kDa protein.
# TABLE OF CONTENTS

**ACKNOWLEDGMENTS** ................................................................................................. ii

**LIST OF TABLES** ........................................................................................................ vii

**LIST OF FIGURES** ..................................................................................................... viii

**INTRODUCTION** ........................................................................................................ 1

- Poxviruses .................................................................................................................... 1
- Virion Structure ............................................................................................................ 2
- Viral Genome Structure and Organization .................................................................. 3
- Viral Infection Cycle ..................................................................................................... 5
- Cytopathic Effect ......................................................................................................... 10
- Poxvirus-Host Interactions .......................................................................................... 11
  - Homologs of the Complement System .................................................................... 13
  - Homologs of Cellular Cytokines and Their Receptors .......................................... 16
  - Inhibition of Tumor Necrosis Factor (TNF) and TNF Receptor Homologs .............. 17
  - Inhibition of Interferon (IFN) and IFN Receptor (IFNR) Homologs ....................... 23
  - Viral Protein Inhibitors of Apoptosis .................................................................... 26
- Tanapox Virus ............................................................................................................... 28
- History ....................................................................................................................... 28
- Virion Structure ........................................................................................................... 29
Table of Contents—continued

Viral Genome Structure and Organization ........................................... 30
Biology.................................................................................................. 31
Host Range ................................................................................... 31
Cytopathic Effect on Host Cell .................................................... 32
Pathogenesis .............................................................................. 33
Growth Kinetics ........................................................................ 35
Anti-Cytokine Activity in Supernatant From TPV Infected Cells ...... 35
Conclusions ................................................................................ 36

MATERIALS AND METHODS ............................................................................ 37
Cells and Virus ............................................................................ 37
Virus Purification ......................................................................... 38
Supernatant Preparation ................................................................ 39
Preparation and Analysis of DNA ................................................... 40
Preparation of TPV Genomic DNA .................................................. 40
Restriction Analysis of DNA ......................................................... 41
Dephosphorylation of pUC 19 ......................................................... 41
S1 Nuclease Digestion of Hairpins ................................................... 42
Repair of S1 Digested Ends ............................................................ 42
Blunt-End Ligation ....................................................................... 43
Transformation ........................................................................... 43
Isolation of Plasmid DNA .............................................................. 44
Table of Contents—continued

Resolution and Recovery of DNA Fragments ............................................... 45

Agarose Gel Electrophoresis .......................................................... 45

Recovery of DNA Fragments .......................................................... 45

Analysis of DNA by Blotting and Hybridization ........................................... 46

Southern Hybridization ........................................................................ 46

DNA Sequencing ............................................................................... 50

Polymerase Chain Reaction (PCR) ..................................................... 50

Cycle Sequencing and Dye Terminator Removal ..................................... 50

Dideoxy Sequencing Method .............................................................. 51

DNA Sequence Analysis ...................................................................... 51

Preparation and Analysis of RNA ......................................................... 52

Preparation of Cytoplasmic RNA ......................................................... 52

Northern Blot Analysis ........................................................................ 53

DNA-Protein Interactions ....................................................................... 53

Preparation of Nuclear and Cytoplasmic Extracts ..................................... 53

Gel Mobility Shift Assay ....................................................................... 54

Analysis and Detection of Proteins ........................................................ 55

Quantitation of Proteins .................................................................... 55

Electrophoretic Separation of Proteins .................................................. 55

Staining Proteins in Gels .................................................................... 56

Drying Gels ....................................................................................... 56

Protein Purification .............................................................................. 56
# Table of Contents—continued

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Sequence Analysis</td>
<td>58</td>
</tr>
<tr>
<td>Enzyme Linked Immunosorbent Assay (ELISA)</td>
<td>59</td>
</tr>
<tr>
<td>RESULTS</td>
<td>60</td>
</tr>
<tr>
<td>Inhibition of CAM Gene Expression</td>
<td>60</td>
</tr>
<tr>
<td>Selective Down-Regulation of TNF-α Induced CAMs</td>
<td>61</td>
</tr>
<tr>
<td>Inhibition of NF-κB Activation and Translocation</td>
<td>64</td>
</tr>
<tr>
<td>TPV Infected Cells Secrete a 38 kDa Polypeptide That Binds to TNF-α</td>
<td>66</td>
</tr>
<tr>
<td>Identification of the 38 kDa Protein</td>
<td>68</td>
</tr>
<tr>
<td>Cloning and Sequencing of the Gene Encoding the 38 kDa Protein</td>
<td>70</td>
</tr>
<tr>
<td>The TPV PstI-J Fragment</td>
<td>70</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>82</td>
</tr>
<tr>
<td>APPENDICES</td>
<td></td>
</tr>
<tr>
<td>A. Biosafety Clearance</td>
<td>88</td>
</tr>
<tr>
<td>B. Radiation Safety</td>
<td>90</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>92</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1. Virus Encoded Host Immunoregulatory Proteins ............................................... 18
2. Hybridization Probes ..................................................................................... 47
3. Hybridization Conditions........................................................................... 49
4. Putative Open Reading Frames of the TPV PstI-J Fragment ....................... 78
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diagram of the Poxviral Structure</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Structure and Organization of the Vaccinia Virus Genome</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Schematic Representation of the Poxviral Infection Cycle</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>The Complement System and Viral Inhibition</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Tanapox Virus</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>Inhibition of CAM Gene Expression by Supernatant From TPV Infected Cells</td>
<td>62</td>
</tr>
<tr>
<td>7</td>
<td>Selective Down-Regulation of CAMs by Supernatant From TPV Infected Cells</td>
<td>63</td>
</tr>
<tr>
<td>8</td>
<td>Inhibition of NF-κB DNA Binding Activity by Supernatant From TPV Infected Cells</td>
<td>65</td>
</tr>
<tr>
<td>9</td>
<td>TPV Infected Cells Secrete a 38 kDa Polypeptide That Binds TNF-α</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>TPV Infected Cells Secrete a 38 kDa Protein</td>
<td>69</td>
</tr>
<tr>
<td>11</td>
<td>TPV-Probe 3 Binds to TPV PstI-J Fragment</td>
<td>71</td>
</tr>
<tr>
<td>12</td>
<td>Sequencing Scheme for the TPV PstI 5.1 kbp J Genomic Fragment</td>
<td>72</td>
</tr>
<tr>
<td>13</td>
<td>DNA Sequence of the 5.1 kbp PstI-J Fragment of the TPV Genome</td>
<td>73</td>
</tr>
<tr>
<td>14</td>
<td>PstI Restriction Map of the TPV Genomic DNA and Location of the Potential ORFs on the PstI-J Fragment</td>
<td>77</td>
</tr>
</tbody>
</table>
INTRODUCTION

Poxviruses

Poxviruses are the largest and most complex of known animal viruses. The family Poxviridae contains two subfamilies: the Chordopoxvirinae, which infect vertebrates and the Entomopoxvirinae, which infect insects. There are eleven genera of the Poxviridae family including the Avipoxvirus, Capripoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus, Yatapoxvirus, and Entomopoxvirus A, B, and C. Viruses included in a genus are related both genetically and immunologically and have a similar ultramicroscopic structure and host range (reviewed in Moss, 1996). Many strains exist that are pathogenic to various host species. The most recognized poxvirus, variola virus, the causative agent of smallpox in humans, is an extremely infectious agent that caused widespread disease. Today, it is mostly of historical significance due to the campaign started in 1966 by the World Health Organization for its worldwide eradication.

Poxviruses have contributed significantly in the advancement of both biomedical and basic molecular biology. In 1796, Edward Jenner used cowpox virus to vaccinate against smallpox. Later, vaccinia virus was used to replace cowpox virus and eventually aided in the eradication of the scourge. In molecular biology, vaccinia virus was the first mammalian virus to be visualized with light microscopy and biochemically characterized. Today, vaccinia virus is used in recombinant techniques (Mackett et al., 1982), where the viral genome provides a vector for the insertion of
foreign genes in vaccine development.

Poxviruses are unique among DNA viruses in that their entire replication cycle occurs in the cytoplasm of host cells. All species except tanapox produce pocks on the chorioallantoic membrane in embryonated chicken eggs (Fenner, 1996).

Virion Structure

Poxviruses have a characteristic oval or "brick-shaped" structure (Figure 1). Analyses of vaccinia virus reveals the dry weight of the virion to be composed of 90% protein, 5% lipid, and 3.2% DNA (Smadel and Hoagland, 1942, Zartouw, 1964). Poxviruses are large, complex particles measuring 300-450 nm x 170-260 nm and can be visualized by a light microscope using the oil immersion lens. Thin sections under

Figure 1. Diagram of the Poxviral Structure. Poxviruses are brick-shaped. A biconcave core, flanked by two lateral bodies, contains the viral genome. Two membranes are found on extracellular, enveloped virions.
electron microscopy reveal an outer surface composed of lipid and proteins surrounding a biconcave or dumbbell-shaped core (DeHarven and Yohn, 1966). The core, studded with 20 nm tubular structures (Moss, 1996), is enclosed by two lateral bodies whose functions are unknown. The double-stranded, linear poxviral DNA is found within the core unit. During replication, 2 forms of viral particles are observed. The intracellular mature virions (IMV) posses a single inner membrane and the extracellular enveloped virions (EEV) (Payne, 1978) contain an additional extracellular or outer lipoprotein envelope.

**Viral Genome Structure and Organization**

Poxviruses are double-stranded, linear DNA viruses with genomes ranging from 130 kilobase pairs (kbp) in *parapoxviruses* (Menna *et al.*, 1979, Robinson *et al.*, 1987) to approximately 300 kbp in *avipoxviruses* (Hyde *et al.*, 1967). Complete nucleotide sequences of vaccinia virus (Goebel *et al.*, 1990) and variola virus (Massung *et al.*, 1993) confirms a conservation of sequences along the central regions and variation in the termini (Mackett and Archard, 1979), where inverted terminal repeats (ITRs) are found. ITRs are identical but oppositely oriented sequences. In variola virus (Figure 2), the ITR sequence is small, approximately 725 base pairs (bp), and contains three 69 bp direct terminal elements, and a 54 bp partial repeat element (Massung *et al.*, 1994). Since inverted terminal repeats may include coding regions, some genes are found at both ends of the genome (Moss, 1996). Proximal to the ITR region, terminal hairpin loops have been identified in vaccinia virus (Garon *et al.*, 1978), variola virus (Massung *et al.*, 1994), cowpox virus (Pickup *et al.*, 1983),
Figure 2. Structure and Organization of the Vaccinia Virus Genome. Poxviral DNA is linear and double-stranded. Terminal loops link both strands at the termini. Inverted terminal repeats are found proximal to the hairpin linked ends. Conserved sequences are centrally located on the genome whereas variable regions exist near the termini. (from Moss, 1996)

Shope fibroma virus (DeLange et al., 1984), swinepox (Massung and Moyer, 1991), and others (Esposito and Knight, 1985). The hairpin loops are largely but incompletely base-paired and extrahelical bases exist on one of the two strands (Baroudy et al., 1982). These genes are variable in poxviruses and may contain genes that are nonessential for replication in tissue culture (Kotwal and Moss, 1988a, Perkus et al., 1991). The terminal regions may therefore also contain numerous genes encoding many potentially novel proteins. Sequencing and computer analysis of the left (52,283 bp) and right (49,649 bp) regions of the cowpox virus revealed 51 and 37 potential open reading frames (ORFs), respectively (Shchelkunov et al., 1998).

Adjacent to the hairpin loop is a region about 100 bp which is highly conserved and essential to the virion (Moss, 1996). Internal to this region exist tandem repeats of short AT-rich sequences which are thought to play important roles in recombinational events.
Poxviral genomes do not contain introns. The promoter regions are short and are found directly upstream of the transcriptional and translational start sites (reviewed in Traktman, 1991). The complete genomic sequence of several poxvirus genomes have been sequenced including the Copenhagen (Goebel et al., 1990) and Ankara (Antoine et al., 1998) strains of vaccinia virus, the India (Shchelkunov et al., 1993b), Bangladesh (Massung et al., 1994), and Western Reserve strains (Earl and Moss, 1993) of the variola virus as well as the molluscum contagiosum virus (Senkevich et al., 1996, Senkevich et al., 1997), the Shope (rabbit) fibroma virus (Willer et al., 1999), the myxoma virus (Cameron et al., 1999), and the *Melanoplus sanguinipes* entomopoxvirus (Afonso et al., 1999). There are approximately 200 genes in the genome.

**Viral Infection Cycle**

Poxviruses are taken up by the host cell through the process of active ingestion or pinocytosis (Joklik, 1968). Viral particles are converted to viral cores in the cytoplasm of the host cell almost immediately following entry to the cell (Joklik, 1964a, Joklik, 1964b, Sarov and Joklik, 1972) (Figure 3). This is the first uncoating process. The viral cores contain the poxviral genome and a pool of proteins including the RNA polymerase, capping/termination protein, poly A polymerase, and early transcription factor (Moss, 1990, Moss, 1996). It is within the viral cores that some early transcription occurs (Kates and McAuslan, 1967, Dahl and Kates, 1970a, Dahl and Kates, 1970b, Polisky and Kates, 1972). These early mRNAs are released from the core and translated on host polysomes (reviewed in Traktman, 1991), resulting in
Figure 3. Schematic Representation of the Poxviral Infection Cycle. The entire replication cycle of poxviruses occurs in the cytoplasm of host cells. Poxviral particles are taken up by the cell through active ingestion. Viral particles are converted to viral cores. A temporal cascade of gene expression follows. Early genes are expressed before viral genome replication and late genes are expressed only after viral DNA synthesis. Poxviral DNA synthesis and assembly of the virus follows. Two forms of the infectious particles can be seen, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV).

an uncoating enzyme that removes the viral coat. In vaccinia virus, early mRNA can be detected within 20 minutes and peak levels can be seen within 2-3 hours (Baldick and Moss, 1993) after viral infection. The transcription of these early genes is regulated by A/T rich sequences, TAAAATG (Broyle and Moss, 1986), that are located immediately upstream, of the RNA start sites (Cochran et al., 1985, Ink and Pickup, 1989, Weir and Moss, 1987). The downstream sequence, TTTT-TAT is the
early transcription termination signal (Amano, et al., 1995). Early proteins produced are crucial for viral DNA replication including thymidine kinase (Dubbs and Kit, 1964, Hruby and Ball, 1982, Weir et al., 1982) thymidylate kinase (Smith, et al., 1989), ribonucleotide reductase (Slabaugh et al., 1988, Tengelsen et al., 1988) and dUTPase (Broyle, 1993).

Viral cores are then broken down following a lag phase dependent on the multiplicity of infection (m.o.i.). This is considered the secondary uncoating step (Sarov and Joklik, 1972) resulting in naked viral DNA. A unique characteristic of poxviruses is their ability to replicate viral DNA independent of the host cell nucleus. This was shown in studies using enucleated BSC-1 cell monolayers infected with vaccinia virus (Prescott et al., 1971, Prescott et al., 1972, Pennington and Follett, 1974). Levels of DNA synthesis were detected in the virus-infected cells despite the absence of a nucleus.

Viral DNA is replicated in an organized area in the cytoplasm (Joklik and Becker, 1964) termed viral factories (Fenner et al., 1989). As infection proceeds, an enlargement of these areas can be seen. The viral genome is inclusive and encodes almost all the proteins necessary for transcription and replication of the virus (Moss, 1992). The timing of DNA synthesis is dependent on the m.o.i., the cell type, and the poxvirus itself. Different members of the poxvirus family will vary in the period to the onset of DNA replication ranging from 1-2 hours post infection (h.p.i.) for vaccinia virus (Joklik and Becker, 1964) to 12-16 h.p.i. for the fowl poxvirus (Prideaux and Boyle, 1987).
DNA replication is postulated to begin with the introduction of a nick in sequences proximal to the genomic terminus (Pogo, 1980, Pogo et al., 1981). The resultant 3’OH primer terminus allows for the viral DNA polymerase to bind. Folding back of this strand on itself enables DNA synthesis throughout the entire genome and around the hairpin termini. It is unknown whether either terminus is preferred. Concatemeric intermediates are formed during replication through the hairpin (Moyer and Graves, 1981). The concatemer junction is made up of an identical copy of the hairpin loop (Merchlinsky et al., 1988). Resolution of the concatemer to unit length molecules occurs during late gene expression (Merchlinsky and Moss, 1989). Studies using temperature sensitive mutants of vaccinia virus with specific inhibitors show that concatemeric forms of DNA accumulate when late gene expression is inhibited (DeLange, 1989, Merchlinsky and Moss, 1989). A main role of DNA replication is in controlling the temporal cascade of gene expression (reviewed in Traktman, 1991). It is DNA replication that induces the switch from early gene synthesis to intermediate gene synthesis. This is a unique feature to poxviruses and the African swine fever virus (Breese and DeBoer, 1966).

Intermediate genes are expressed following DNA replication but prior to late gene expression (Moss and Salzman, 1968, Pennington, 1974, Vos and Stunnenberg, 1988). Few intermediate genes have been identified. Three known intermediate genes encode transactivators of late genes (Keck et al., 1990). The promoter sequence of intermediate genes contains an initiator element, TAAA (Baldick et al., 1992). Intermediate mRNA levels in vaccinia virus peak at 100 minutes post-infection (Vos and Stunnenberg, 1988).
Late gene expression begins on the derepression of intermediate genes. In vaccinia virus, late stage mRNA can be detected at 140 minutes post-synchronous infection. Late genes are expressed only after genome replication since late promoters are dependent on DNA replication for activity. Late genes synthesize proteins involved in major virion structural components as well as virion enzymes needed early during infection. The late genes are found throughout the genome but cluster in the central region (Belle Isle et al., 1981). An initiation sequence of TAAATG/A is found at the start of late open reading frames (ORFs) (Davison and Moss, 1989b).

Assembly of the virus, as replication, occurs in the cytoplasm of infected cells. Two distinct forms of infectious virus particles, intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Payne, 1978), can be seen during morphogenesis. The majority of the virus particles generated are IMV with EEV only representing 1-10% of the total virus progeny in vaccinia virus (Payne, 1980). Both forms are infectious but EEV appears to have a higher infectivity (Boulter and Appleyard, 1973). IMV derive a double membrane from the trans-Golgi network to form EEV. EEV migrate to the cell surface along actin-containing microfilaments (Hiller et al., 1981). At the cell surface, EEV fuses its outer layer with the host cell plasma membrane. Virions are subsequently released by budding (Payne, 1978).

Poxviral infection in tissue culture, halts host macromolecular synthesis. Inhibition of host protein synthesis is well documented in the vaccinia virus (Moss and Salzman, 1968). The time course of protein synthesis inhibition varies between 8-16 h.p.i. among poxviruses (Harper et al., 1979). However, the majority of host
protein synthesis continues for as long as 72 h.p.i. in Yaba-infected cells (Vafai and Rouhandeh, 1982).

Cytopathic Effect

The cytopathic effect that ensues poxvirus infection varies with virus strain and cell type. In general, morphological cytopathogenesis involves the rounding of infected cells followed by cytoplasmic granulation. In some cases, cell-cell fusion has also been reported (reviewed in Traktman, 1991). Poxviral infection induces the host cell to display a rapid alteration in cell shape and cytoskeletal reorganization (Traktman, 1991). Microscopic observations also show that cytoplasmic inclusions are correlated with poxvirus infection, as infection proceeds, an enlargement of these areas can be seen. At the molecular level, infection of tissue culture cells with vaccinia virus or other orthopoxviruses results in changes in membrane permeability (Carrasco and Esteban, 1982) and inhibition of host DNA, RNA, and protein synthesis (Moss, 1996).

Some poxviruses including the Shope fibroma virus (Shope, 1932), Yaba virus (Bearcroft and Jamieson, 1958), and molluscum contagiosum virus (Postlethwaite, 1970) induce hyperplasia and even tumors in the skin of their hosts. A nonessential gene encoding a homolog of epidermal growth factor (EGF) and transforming growth factor-α was discovered in vaccinia virus which was attributed to the development of the hyperplastic response (Blomquist et al., 1984, Twardzik et al., 1985, Buller et al., 1988b). *In vivo* experiments revealed that infection with wild-type virus caused rapid proliferation of ectodermal and endodermal cells of chicken
embryo chorioallantoic membrane, whereas this was not seen in mutant virus lacking the VGF gene (Buller et al., 1988a).

Poxvirus-Host Interactions

Viruses, in the most general sense, are obligate intracellular parasites. They rely on their hosts for sources of energy and protein synthesis and are capable of manipulating host cellular machinery to their needs. The host however possesses an arsenal of defense molecules, in the form of the immune system which is efficient in destroying inhibiting, and disabling the virus. The survival of the virus *in vivo* therefore depends on its capacity to evolve modes of evasion, suppression, or inactivation of the host immune system. The virus and host consequently engage in a form of combat, once cleverly referred to as a form of "Star Wars" (Barinaga, 1992). Survival and success of both the host and virus are at stake.

Viruses use a vast array of strategies to evade the immune response. RNA viruses such as rhinovirus (Hayder and Mullbacher, 1996) and influenza virus (Wiley *et al.*, 1981) utilize antigenic variation as a means to escape host immune components. This property allows the viruses to exist in multiple antigenically distinct strains. Rhinoviruses and influenza viruses therefore depend on non-immune hosts and rapid transmission from host to host for their survival. The human immunodeficiency virus (HIV) also utilizes a similar strategy of constant antigen evolution to evade the immune system (Coffin, 1995). HIV however, can at any one time exist in several dozen different antigenic forms within a single infected individual. Other viruses such as the poxviruses (Palumbo *et al.*, 1994, Smith, 1993,
Pickup, 1994, McFadden et al., 1995), adenoviruses (Wold and Gooding, 1991, Hayder and Mullbacher, 1996), and herpesviruses (Hsu et al., 1990, Bhat and Thimmappaya, 1983, Poynter-Davis and Farrell, 1996), which are antigenically stable, use virally encoded protein homologs of host immune factors to evade the immune response. These viral homologs are most likely genes acquired from their hosts.

In the past 10 years, there has been a great advancement in the understanding of these virally encoded proteins for defense. To date a large set of viral protein factors that contribute to virulence have been identified. Modulators of the host immune response include inhibitors of complement control proteins, apoptosis, inflammation, and cytokine-receptor mimicking molecules. These immunomodulators are virally encoded proteins possessing sequence similarity with host immune factors. Members of the poxvirus family were the first DNA viruses shown to encode secreted versions of cellular cytokine receptors (Smith et al., 1991).

Virokines (Kotwal and Moss, 1988b) and viroceptors (Upton et al., 1991) are two terms coined to classify groups of viral defense molecules. Virokines are a group of virally encoded proteins secreted from infected cells which function by mimicking extra-cellular signaling molecules such as cytokines or other secreted regulators (McFadden, 1995). Virokines act to antagonize the effects of host signaling molecules involved in the anti-viral response. Viroceptors are similar to virokines but mimic cellular receptors and act by diverting cytokines from binding their normal receptors. Both virally encoded proteins function to basically interrupt the "immune circuitry" (McFadden, 1995) and provide the virus a means to propagate.
Immunomodulating viral polypeptides may have potential therapeutic actions, especially in autoimmune diseases, since they are capable of inactivating or inhibiting cytokine activities. This review will encompass a wide array of virulence factors but will focus primarily on poxvirus encoded virulence factors.

Homologs of the Complement System

The complement system is a highly regulated series of 20 functionally linked proteins (Figure 4). The system functions principally in (a) cytolysis of foreign organisms, (b) opsonization which enhances phagocytosis, and (c) activation of inflammation by serving to promote chemotaxis of inflammatory cells and stimulate the release of chemical inflammatory mediators from leukocytes (for review see Muller-Eberhard, 1988, Reid and Day, 1989).

Two activation pathways exist for the complement system (Figure 4). The first, the classical pathway, is activated by antibody-antigen complexes and the second, the alternative pathway, is activated directly by microbial surfaces in the absence of antibody. Although differing in their modes of activation, both pathways share many late stages and effector functions. Their functions converge and lead to the formation of the membrane attack complex (MAC) which induces the osmotic lysis of foreign or virally infected cells (Muller-Eberhard, 1986). Complement-mediated lysis is an important component of the host defense against microbial infection including viruses.
Antigen-Antibody Complex

C1 → Activated C1 → C4 + C2 → C4b2a → C4b2a3b

C3 → C3b → C3bBb → C3bBb3b

Factor D

Factor B

Microbial Surfaces
Polysaccharides

MEMBRANE ATTACH COMPLEX (MAC)
C5-9

CLASSICAL PATHWAY

VCP

ALTERNATIVE PATHWAY

=Herpes virus gC protein

=Vaccinia virus complement control protein

= Herpes virus saimiri-15 protein

Figure 4. The Complement System and Viral Inhibition. Viruses have evolved various mechanisms to counteract the activities of the host complement system. Surface glycoprotein C (gC) of the herpes simplex virus-1 and -2 functions as a C3b receptor. The γ-herpes virus saimiri protein 15 (HVS15) prevents complement mediated cytolysis by inhibiting the assembly of the membrane attack complex (MAC). The vaccinia virus complement control protein (VCP) binds C4 and inhibits the classical pathway.
Viruses have evolved measures to counteract the activities of the complement system (Figure 4). Surface glycoprotein C (gC) of the herpes simplex virus-1 (HSV-1) and -2 (HSV-2) functions as a C3b receptor. C3b is normally induced on cells following HSV infection. C3b is critical in both the classical and alternative pathways of the complement system. Binding of the C3b component of the complement pathway to HSV-gC prevents complement lysis and virus neutralization (Friedman et al., 1984, Fries et al., 1986, McNearney et al., 1987, Harris et al., 1990). The HSV-gC thereby very effectively inhibits both alternative and classical pathways (Harris et al., 1990).

The gamma herpes virus saimiri (HVS) encodes two proteins, HVS 04 and HVS 15 that interfere with complement activity. HVS 04 inhibits the classical pathway (Fodor et al., 1995) by inhibiting C3-convertase activity. Fodor et al. (1995) demonstrated that cells transfected with HVS 04 were resistant to complement-mediated cytolysis. This was attributed to the functional homology of HVS 04 to decay accelerating factor (DAF), a protein which functions to inhibit the assembly of C3 convertase. HVS 15 acts to prevent complement-mediated cytolysis by displaying a strong homology to a complement component, CD59. CD59 inhibits assembly of MAC by blocking C9 (Rother et al., 1994). By blocking the MAC, HVS potentially enhances virus survival by inhibiting the destruction of virally infected cells.

The vaccinia virus complement-control protein (VCP) binds the C4b fragment of complement component C4 (Kotwal and Moss, 1988b). VCP is a major 35 kDa secretory protein that is structurally similar to the C4-binding protein. VCP was shown to inhibit hemolysis of sensitized sheep erythrocytes in human serum by
interfering with complement-mediated attack (Kotwal et al., 1990). VCP inhibits complement fixation mediated by the classical pathway but not the antibody-independent alternative pathway (Kotwal et al., 1990). Animal studies show that there is decreased virulence in mutant viruses lacking the gene encoding VCP (Kotwal et al., 1990). By inhibiting the complement system, the virus can circumvent one of the most important host responses for virus neutralization and cytolysis of virus-infected cells.

Homologs of Cellular Cytokines and Their Receptors

Cytokines are protein molecules involved in the effector phases of both natural and specific immunity. Cytokines are crucial for communication between cells of the immune and inflammatory systems. In general, cytokines are synthesized in response to inflammatory or antigenic stimuli and act locally in an autocrine or paracrine fashion, by binding to high affinity receptors found on target cells.

Multiple and diverse cell types produce cytokines which themselves are pleotropic and can act on various cells. The actions of cytokines can be redundant and often influence the synthesis of other cytokines as well as their own actions. Cytokines serve many functions that are critical in the host immune response against pathogens and also regulate the magnitude and nature of the immune response. Cytokines are an integral component of the host response to viruses. For example, TNF-α is crucial in antiviral activity by mediating the lysis of infected cells whereas interferons act to protect uninfected cells from virus infection. Several viruses have
acquired homologs of cytokines or cytokine receptor genes that they use to manipulate the host cytokine network (Table 1).

Inhibition of Tumor Necrosis Factor (TNF) and TNF Receptor Homologs

Activated mononuclear phagocytes and T-cells synthesize TNF as a nonglycosylated transmembrane protein of approximately 25 kDa. A 17 kDa fragment at the carboxy terminus, is proteolytically cleaved off the plasma membrane to produce the secreted form, which circulates as a stable homotrimer of 51 kDa. TNF exists in two homologous forms, TNF-α and TNF-β.

Binding of the soluble TNF trimer to its cellular receptor initiates the activity of this cytokine. TNF receptors are present on almost all cells. There are two distinct TNF receptors of 55 kDa (p55) and 75 kDa (p75). They are expressed independently on the membranes of cells and share 28% homology. Both TNF receptors bind TNF-α and TNF-β. The receptors share no homology however in their intracellular domains implying that they utilize different signaling pathways (Dembic et al., 1990). They are members of the NGF/TNF receptor superfamily which includes CD27, CD30, CD40, and Fas antigen (Itoh et al., 1991, Camerini et al., 1991). These receptors are defined by multiple cysteine-rich domains in the extra-cellular region, each containing about 40 amino acids (Smith et al., 1994).

TNF is the principal mediator of the host response to infectious organisms, including bacteria. It affects nearly every tissue and organ system (reviewed in Dinarello, 1992). The biological actions of TNF include: (a) the upregulation of cell
Table 1

Virus Encoded Host Immunoregulatory Proteins

<table>
<thead>
<tr>
<th>Virus Family</th>
<th>Virus</th>
<th>Product</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>E3-14.7k</td>
<td>Inhibits TNF-induced cytolysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E3-10.4k/ E3-14.5k</td>
<td>Inhibits TNF-induced cytolysis, down-regulates EGFR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E1B-19k</td>
<td>Inhibits TNF-induced cytolysis, blocks p53</td>
<td></td>
</tr>
<tr>
<td>Herpesvirus</td>
<td>HSV-1</td>
<td>gC</td>
<td>C3b receptor homolog, inhibits complement lysis</td>
</tr>
<tr>
<td></td>
<td>HSV-2</td>
<td>gC</td>
<td>C3b receptor homolog, inhibits complement lysis</td>
</tr>
<tr>
<td></td>
<td>γ-HVS</td>
<td>HVS 04</td>
<td>Inhibits C3 convertase, prevents complement lysis</td>
</tr>
<tr>
<td></td>
<td>EBV</td>
<td>BhRF1</td>
<td>Blocks C9, inhibits MAC assembly</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bcl-1 homolog, inhibits apoptosis</td>
</tr>
<tr>
<td>Poxvirus</td>
<td>vaccinia virus</td>
<td>E3L</td>
<td>Inhibits IFN-γ activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K3L</td>
<td>Blocks phosphorylation of eIF2-α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VCP</td>
<td>Binds C4b and inhibits complement lysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C22L/ B28R</td>
<td>TNF-homolog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B18R</td>
<td>IFN-homolog</td>
</tr>
<tr>
<td></td>
<td>SFV</td>
<td>T2</td>
<td>TNF homolog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T7</td>
<td>IFN-homolog</td>
</tr>
<tr>
<td></td>
<td>myxoma virus</td>
<td>M-T2</td>
<td>TNF homolog, inhibits apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M-T7</td>
<td>IFN-homolog</td>
</tr>
<tr>
<td></td>
<td>cowpox virus</td>
<td>CrmB</td>
<td>TNF-homolog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CrmC</td>
<td>TNFRII-homolog</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CHOhr</td>
<td>Inhibits apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SPI-2/CrmA</td>
<td>Inhibits apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CrmA</td>
<td>ICE-homolog</td>
</tr>
</tbody>
</table>
adhesion molecules on vascular endothelial cells which are essential for the binding of leukocytes at sites of inflammation and infection, (b) the activation of inflammatory leukocytes including morphonuclear leukocytes, (c) the stimulation of mononuclear phagocytes and other cell types to produce cytokines, including IL-1 and TNF itself, and (d) the cytolysis of cells infected with certain viruses (reviewed in Beutler and Van Huffel, 1994, Beutler and Cerami, 1989).

With the pleotropic nature of TNF, it is not surprising that viruses have evolved mechanisms to suppress its activity. Adenoviruses are non-enveloped, DNA viruses. The double-stranded DNA is transcribed in two phases, early and late, in the nucleus of infected host cells. Among the 20-25 adenovirus early genes, there are at least 4 gene products with anti-TNF activity. The E3-14.7k, E3-10.4k, and E3-14.5k proteins are transcribed from the E3 region of the viral genome while the E1B-19k is transcribed from the E1 region (reviewed in Wold and Gooding, 1991). These proteins act to prevent TNF-induced cytolysis employing distinct mechanisms. E3-10.4k and E3-14.5k are transmembrane proteins present in the plasma membrane (Hoffman et al., 1992) that function together as a heterodimer (Tollefson et al., 1991). Hoffman et al. (1992) showed that in addition to inhibiting TNF-induced cytolysis, that the E3-10.4k and E3-14.5k heterodimer further function to down-regulate the epidermal growth factor receptor (EGFR). The E3-14.7k product is a 128 amino acid protein found in the nucleus and cytoplasm of infected cells (Persson et al., 1979) whereas the E1B-19k protein (Gooding et al., 1991) is found in the nuclear envelope of infected cells. Unlike E3-10.4k and E3-14.5k, both E3-14.7k and E1B-19k act independently to inhibit TNF activity. By conferring TNF resistance and EGF down-
regulation to the virus, these four adenovirus proteins allow the adenovirus to escape one component of the host immune response.

Several poxviruses have also acquired anti-TNF activity in the form of homologs of the TNF receptor. These homologs are capable of binding TNF and nullifying its activity. The Shope fibroma virus (SFV) is a member of the *leporipoxviruses*. It is a tumorigenic virus which induces invasive malignancies in immunocompromised rabbits and benign fibromas in adults rabbits (reviewed in McFadden, 1988). SPV encodes a soluble receptor of the type I TNF receptor, termed T2 (Smith *et al.*, 1991). The T2 gene was isolated from the ITR regions of the SFV genome and found to be an early gene (Upton *et al.*, 1987). Recombinant T2 protein, expressed in COS cells, was secreted as a soluble glycoprotein with an apparent molecular weight of 58 kDa (Smith *et al.*, 1991). The cytoplasmic domain of the T2 protein contains 129 amino acid residues with no sequence homology to any known proteins. In TNF binding assays, T2 was shown to bind specifically to mouse and human TNF-α and TNF-β, inhibit the binding of TNF-α and TNF-β to their native cellular receptors, and inhibit TNF-mediated cytolysis of murine L929 fibroblasts (Smith *et al.*, 1991). Statistical analysis comparing the T2 sequence with those of all cellular TNF receptor family members established T2 as an acquired p75, not p55, TNF receptor (Smith *et al.*, 1990, Smith *et al.*, 1991). T2 was the first soluble cytokine receptor, specifically p75 TNF receptor, to be identified in a virus.

The myxoma virus, a pathogenic virus of European rabbits, which induces an often fatal systemic infection (reviewed in McFadden *et al.*, 1997), was subsequently found to possess a TNF-binding protein. Upton *et al.* (1991) identified two copies of
an M-T2 gene, one on each of the terminal regions of the myxoma genome. The M-T2 protein was shown to have significant homology to the ligand binding domain of the TNF receptor gene family (Upton et al., 1991). When both copies of the M-T2 gene are inactivated, a dramatic disease attenuation is seen in infected rabbits. The majority of the rabbits infected with the M-T2-knockout virus were able to mount an effective immune response and recover from the otherwise fatal infection (Upton et al., 1991). M-T2 is secreted from virally infected cells as both a monomer of 55-95 kDa and as a 90 kDa disulfide-linked dimer, both of which bind TNF-α with similar affinities (Schreiber et al., 1996). The dimer however is a far more potent inhibitor of TNF-α. The C-terminal contains a 132 amino acid sequence that is distinct and unique to myxoma, although it is relatively conserved in some of the other poxviral TNF homologs. The M-T2 protein inactivates TNF in a species specific manner (Schreiber and McFadden, 1994). The cytolytic activities of rabbit TNF-α but not mouse or human TNF-α are inhibited by M-T2. Scatchard analysis of the binding specificities of M-T2 (Schreiber et al., 1996) revealed that M-T2 binds to rabbit TNF-α with an affinity which is comparable to that of cellular receptors. These studies all demonstrate that M-T2 is a powerful virulence factor of myxoma virus contributing to viral pathogenesis. It also further indicates the importance of TNF-α in viral infections.

The cowpox virus, a member of the orthopoxviruses, was found to encode two soluble homologs of the cellular TNF receptor termed CrmB and CrmC (Smith et al., 1996). The crmB gene was identified in the ITR of the Brighton Red strain of the
cowpox virus (Hu et al., 1994). The crmB gene is transcribed from an early promoter. Its primary product is a 355 amino acid protein containing a signal peptide sequence and three potential N-linked glycosylation sites. The mature gene product is secreted with an approximate molecular mass of 48 kDa. The CrmB protein can bind TNF-α and TNF-β (Hu et al. 1994). Analysis of the N-terminal sequence further revealed close sequence homology with the human TNF receptor Type II. The 161 amino acid C-terminal revealed no similarity to any human TNF receptor. The CrmB protein shows 48% identity to leporipoxviruses and 85% identity to the G4R/G2R ORF of variola virus. The crmC gene is a single copy gene and unlike crmB, is expressed late during viral infection. The 186 amino acid secreted protein, CrmC, is cysteine-rich, has two potential glycosylation sites, and an N-terminal, which constitutes a signal peptide. A 25 kDa recombinant form of CrmC has been shown to bind TNF specifically and completely inhibit TNF-mediated cytolysis. The crmC gene has the strongest homology to the TNF type two receptor gene. Unlike the CrmB protein, the CrmC protein does not bind TNF-β and lacks the conserved C-terminal domain of CrmB proteins.

Vaccinia virus is the prototype of poxviruses. It has a 190 kbp genome, which has the capacity to encode over 200 proteins (Essani and Dales, 1979). The vaccinia virus equivalent of the TNF receptor homolog shows a disruption in the ORF (Howard et al., 1991). The introduction of downstream reading frame shifts however allows the 122 residue ORF, originally designated C22L (repeated as B28R in the right ITR), to be assembled into almost a full complement of four cysteine-rich
pseudorepeats. Connection of three additional ORFs downstream from C22L completes a coding region strongly homologous to the C-terminal element of T2.

The poxviral TNF receptor homologs are all located in the ITRs, near the genomic termini. All TNF receptor homolog ORFs, except in vaccinia virus, are encoded by a continuous nucleotide sequence. All the ORFs however have remarkable sequence conservation in both the N-terminal, which is cysteine-rich, and the C-terminal regions. Variola virus and cowpox virus, both orthopoxviruses, share 85% identity and SFV and myxoma virus, both leporipoxviruses, are similarly conserved (reviewed in Hu et al., 1994). The N-terminal sequence is conserved although the C-terminal domain varies slightly between the viruses. M-T2 and SPV-T2 are transcriptionally early genes. The main function of these poxviral homologs is viral inhibition of host-produced TNF. The viral TNF receptor homologs have structural and functional similarity to cellular TNF receptors that are probably their evolutionary origin.

**Inhibition of Interferon (IFN) and IFN Receptor (IFNR) Homologs**

Gamma interferon (IFN-γ), also called type II interferon, is produced by both IL-2 secreting CD4+ helper T-cells and almost all CD8+ T-cells. IFN-γ is crucially involved in inducing an anti-viral state (Isaacs and Lindenmann, 1957). This is primarily a paracrine function, in that virally infected cells secrete IFN-γ to protect neighboring cells not yet infected and thereby preventing viral proliferation. IFN-γ is also a potent activator of natural killer cells and the lytic potential of these cells which
is crucial in destroying virally infected cells (Isaacs and Lindenmann, 1957). IFN-γ acts to increase the expression of class I MHC molecules, which are recognized by cytolytic T-lymphocytes (CTL). In addition, IFN-γ is also a potent stimulator of mononuclear phagocytes and directly promotes differentiation of T- and B-lymphocytes (reviewed in McNair and Kerr, 1992). IFN-γ is also involved in the activation of vascular endothelial cells, promoting CD4+ T-cell adhesion. It can be seen that IFN-γ therefore is critical in the elimination of viral infection and in the inflammatory response.

IFN-γ itself does not directly prevent viral infection, but inhibits viral spread by induction of anti-viral pathways within the immune system (reviewed in McNair and Kerr, 1992). PKR, a double-stranded RNA-dependent protein kinase, and 2-5A-synthetase are two enzymes induced by all IFNs (Farrell et al., 1977, Kerr and Brown, 1978). Activated PKR phosphorylates the α-subunit of the eukaryotic translation initiating factor, eIF, an important component of cellular protein synthesis. Phosphorylated eIF remains in a complex with eIF2β, preventing its recycling, and thereby shutting down protein synthesis. The vaccinia virus E3L and K3L intracellular proteins (Chang et al., 1992, Davies et al., 1992) block RNA activation and phosphorylation of eIF2-α, respectively. HIV and SIV have been shown to down-regulate PKR, poliovirus has been shown to degrade PKR and EBV, reovirus and influenza virus have been shown to inhibit activation of PKR (reviewed in Mathews, 1993).
Poxviruses are, to date, the only viruses shown to encode IFN-binding proteins. The myxoma virus encodes an IFN-\(\gamma\)R homolog, designated MT7, which is found within the ITR regions of the viral genome (Upton et al., 1992). The MT7 gene product is a 37 kDa soluble protein with sequence homology to both murine and human IFN-\(\gamma\)Rs (Upton et al., 1992). The protein is expressed during early and late times of infection. Mossman et al. (1995c) determined the prevalence of the MT7 protein throughout viral infection to be attributed to the stability of the viral mRNA and not due to the constitutive expression of the viral mRNA. The MT7 protein is secreted by 4 hours post-infection and is species-specific (Mossman et al., 1995c). The MT7 protein only binds and inhibits the activity of rabbit IFN-\(\gamma\)R not human or mouse.

SFV also encodes an IFN-\(\gamma\)R homolog, denoted ST2 (Upton et al., 1991) that has been mapped to the SFV-ITRs (Upton et al., 1987, Upton et al., 1992). The ST2 gene product is a soluble protein composed of 265 amino acids and has 68% homology to the MT7 gene. The ST7 protein is not expressed at levels as high as MT2 although it is present at both early and late times of infection.

The B18R gene product of the Western Reserve (WR) strain of the vaccinia virus binds type I IFN and blocks IFN-\(\gamma\) transmembrane signaling (Goebel et al., 1990). The B18R protein binds human IFN-\(\gamma\) with a high affinity and cells infected with the WR strain of the vaccinia virus, with an inactivated B18R gene, failed to bind radioiodinated IFN-\(\gamma\) (Colamonici et al., 1995). B18R exists as a soluble form (Alcami and Smith, 1995) and as a bound form (Ueda et al., 1990). In competition
experiments, Colamonici et al. (1995) showed that 100µl of medium containing the B18R gene product blocked binding of IFN-γ to the human receptor. The B18R gene product interestingly was shown to neutralize the activity of human as well as rat (Alcamì and Smith, 1995) and chicken (Puehler et al., 1998) IFN-γ. Since the origins of vaccinia virus are still unclear, such findings bring us closer to determining its origins.

With the increasing knowledge of poxviral sequences, other IFNR homologs are now being determined including the B8R of variola virus, Bangladesh-1975 strain, B9R of the variola virus, India-167 strain (Massung et al., 1993, Shchelkunov et al., 1993), and the C6L protein of the swinepox virus (Massung et al., 1993). Ectromelia virus of mice and the cowpox virus have also been identified to encode IFN-γR homologs (Mossman et al., 1995b). Comparison of the poxviral IFN-γR homologs reveal a conservation of 8 cysteine residues in the MT2, ST2, and C6L proteins and 6 in the B8R and B9R proteins (Mossman et al., 1995b). All poxviral IFN-γR homologs are secreted from infected cells and lack the transmembrane domains (Mossman et al., 1995b). They all share significant homology to the human IFN-γR but the extent of homology with the entire sequence is low with only 20-25% identity (Mossman et al., 1995b).

Viral Protein Inhibitors of Apoptosis

Apoptosis or programmed cell death is an important component of the host immune response against viral infection. Virally infected cells can undergo "suicide"
to limit viral multiplication. Viruses in turn have evolved mechanisms to inhibit apoptosis. The Epstein-Barr virus (EBV) BhRF-1 protein shows homology to Bcl-1, a cellular protein inhibitor of apoptosis (Henderson et al., 1993, Tarodi et al., 1994). BhRF-1 was shown to successfully protect human B-cells from undergoing apoptosis (Henderson et al., 1993). The African swine fever virus LMW5-HL gene also shows homology to Bcl-2 and BhRF-1 (Neilan et al., 1993). The adenovirus E1B-19k protein has sequence similarity to Bcl-2 (Rao et al., 1992, Tarodi et al., 1993) and inhibits apoptosis (White et al., 1991, Rao et al., 1992) by blocking p53, a well known tumor suppressor protein which induces apoptosis (reviewed in Levine, 1993). Several poxviruses have also been shown to encode anti-apoptotic genes including the CHOhr of the cowpox virus (Ink et al., 1995), the SPI-1 from rabbitpox virus (Ali et al., 1994), the SPI-2/crmA of the cowpox virus (Gagliardini et al., 1994, Ray et al., 1992), and the myxoma virus, MT2 and M11L (Macen et al., 1996, Upton et al., 1987).

As more information on viral genes is generated through the growing DNA sequence databases, new knowledge will be gained on the multi-functionality of many of the known viral protein homologs of the host immune system. As with the MT2 and the crmA genes, which were thought once to encode a TNFR homolog (Upton et al., 1987) and interleukin-1β converting enzyme (ICE) (Ray et al., 1992) respectively, and now also believed to participate in anti-apoptotic roles (Tewari and Dixit, 1995, Schreiber et al., 1997).
Tanapox Virus

Tanapox virus (TPV), Yaba virus, and Yaba-like disease virus (YLDV) are members of the *Yatapoxvirus* genus based on their size, shape, and ultrastructure. *Yatapoxviruses* infect all primates but cause disease primarily in non-human primates. *Yatapoxviruses* are antigenically related to one another but not to monkeypox or vaccinia viruses (Niven *et al.*, 1961, Downie *et al.*, 1971). Due to similarities in serological tests between TPV and YLDV, YLDV has been referred to in the literature as being identical to TPV (Downie and Espana, 1972). However, restriction maps (Knight *et al.*, 1989) reveal that TPV and YLDV are clearly distinct viruses.

History

The first occurrence of TPV disease was in Ngua, an island swamp forest in Kenya, Africa, in 1957 when a small group of school children developed disease associated later with TPV. The virus was not isolated however until 1962 when a larger outbreak occurred in a population living along the Tana River Valley in Kenya, Africa (Downie *et al.*, 1964). Outbreaks are now known to be endemic in the areas of Kenya and Zaire (Axford and Downie, 1979). In Kenya, the epidemics seen in 1957 and 1962 were associated with periods of extensive flooding (Downie, 1971). In Zaire cases are seen throughout the year but mostly between November and March (Jezek *et al.*, 1985). Most of these cases occurred in people living along the Zaire river.
Virion Structure

TPV is similar to other poxviruses in that virions are enveloped with a brick-shaped or oval morphology (Figure 5) are large in size ranging from 280 x 200nm and can be visualized by the best light microscope (Downie et al., 1971). Thin sections under electron microscopy reveal an outer surface composed of lipid and proteins surrounding a biconcave or dumbbell-shaped core (Knight et al., 1989). The core is enclosed by two lateral bodies (Knight et al., 1989) whose functions are unknown. The double-stranded, linear poxviral DNA is found within the core unit.

Figure 5. Tanapox Virus. Electron micrograph of purified tanapox virus stained with uranyl acetate. Bar = 100nm
Viral Genome Structure and Organization

TPV is a double-stranded, linear DNA virus. The genome of TPV, like that of YLDV (Knight et al., 1989) and Yaba virus (Kilpatrick and Rouhandeh, 1985, Kilpatrick and Rouhandeh, 1987), is approximately 145 kbp as determined by restriction endonuclease analysis (Knight et al., 1989). Although extensive cross-hybridization has been documented between the three viruses (Knight et al., 1989), the TPV genome is distinct from Yaba virus and YLDV. ITRs probably exist at the two ends of the genome since they have been reported in most other poxviruses (reviewed in Moss, 1996). Proximal to the ITR region, terminal hairpin loops have been identified (Kilpatrick and Rouhandeh, 1987, Knight et al., 1989). These genes are variable in poxviruses and may contain genes that are nonessential for replication in tissue culture (Kotwal and Moss, 1988a, Perkus et al., 1991). The terminal regions may therefore also contain numerous genes encoding many potentially novel proteins.

About 17% of the TPV genome has been cloned (Neering, masters thesis, 1993). TPV PstI digested DNA fragments: F (9.0 kbp), H (6.6 kbp), J (5.1 kbp), L (2.1 kbp), M (1.4 kbp), and N (1.1 kbp), were successfully shotgun cloned into pUC 19. The PstI-L fragment was completely sequenced, predicted amino acids and putative open reading frames (ORFs) analyzed by Neering (1993). Intelligenetics analyses revealed 37 complete ORFs and 1 partial ORF within this fragment.
Biology

Downie et al. (1964) first recovered TPV from human skin lesions and cultured the virus in vervet-monkey kidney cells. TPV has since also been successfully cultured in primary human amnion, human embryo lung cells (WI-38), human thyroid cells, vervet monkey kidney cells (BSC-1), patas monkey cells, (Downie et al., 1971), embryonic rhesus monkey kidney cells (EMK) (Espana et al., 1971), CV-1 African green monkey kidney cells (Knight et al., 1989), owl monkey kidney (OMK) (Essani et al., 1994), and Vero cells (Downie and Espana, 1972). The virus cannot be cultured in non-primate cell lines. No cytopathic changes were seen in tissue cultures of chick embryo fibroblasts, primary rabbit kidney, or primary cultures of bovine embryo kidney (Downie et al., 1971) infected with TPV. Another interesting aspect of TPV is that unlike other poxviruses, TPV does not produce pocks on chick chorioallantoic membrane (Downie et al., 1971).

Host Range

Monkeys are the only laboratory animals proven susceptible to TPV infection. Downie et al. (1971) could not transmit TPV disease to a calf, a lamb, young pigs, a goat, guinea pigs, mice or rabbits. No neutralizing antibodies were detected in sera from any of these animals either. However, the same virus suspension (4x10^6 ffu/ml titer) used for inoculum of the above animals did produce papules at the site of inoculum in 2 rhesus monkeys and 2 vervet monkeys. Neutralizing antibodies were detected in the sera of the monkeys (Downie et al., 1971). In studies on a human volunteer, vaccinated against smallpox, nodules were seen at the sites of intradermal
injections and a pock-like lesion with a 1cm diameter was seen at the site of scarification by day 7 (Downie et al., 1971). Poxviral particles were found upon electron microscopy examination. No neutralizing antibodies were found in the sera of the human volunteer. These studies demonstrate the restricted range of hosts for TPV to be either man or monkey.

**Cytopathic Effect on Host Cell**

Cytopathic changes resulting from TPV infection are seen 10 days after TPV inoculation in primary human thyroid cells (Downie et al., 1971) and BSC-1 cells (Downie and Espana, 1972), and 5-6 days after inoculation in Vero cells (Downie et al., 1971). This is slightly faster than in Yaba virus infected cells where plaques cannot be detected until at least 10-12 days (Yohn et al., 1966). Generally, cytopathic changes of TPV include increased granulation of the cytoplasm, rounding up of cells, and eventual destruction of the cell monolayers as viral infection proceeds. According to studies using electron microscopy by Espana et al. (1971), it was also found that TPV causes "focal lesions" characterized by enlargement of the nuclei of infected cells and more densely staining nucleoli. Nicholas and McNulty (1968) also noted nuclear vacuoles after 6 days of TPV infection. The significance of these nuclear vacuoles however is not known and has not been noted in Yaba or monkeypox virus infected cells (Nicholas and McNulty, 1968). The cytoplasm of TPV infected cells contained many poxvirus particles in different stages of development in viral factories (Espana et al., 1971). Espana et al. (1971) identified two types of cytoplasmic inclusions. The first was approximately 1µm in diameter,
composed of finely granular material and viral membranes with spicules were observed. The second type of inclusion was larger and contained more coarsely granulated materials. Espana et al. (1971) postulated that the first inclusion appeared to be the site of virus membrane manufacture and the second inclusion area the site of the development of the nucleoids, presumably DNA.

Plaque assays using BSC-1 by Nicholas and McNulty (1968) show that plaques are less than 1mm in diameter and do not appear until 4-6 days post infection. Knight et al., (1989) found similar sizes in plaques seen in CV-1 cell monolayers. Cells and medium sonicated to release virus, at the time of maximum CPE, yielded $10^5$-$10^6$ pfu/ml cells. Concentration and purification of the virus using sucrose density gradients increased the total virus numbers to $10^7$-$10^8$ pfu/ml (Nicholas and McNulty, 1968).

Pathogenesis

In humans, TPV causes mild, self-limiting disease characterized by swelling of regional lymph nodes, transient fever, headaches, and pock-like skin lesions (Downie et al., 1971). TPV induced skin lesions are similar to the pock-like lesions of smallpox but are larger and develop slower (Downie et al., 1971). The pock-like lesions are initially nodular, resembling an insect bite, and gradually enlarge to form papules with a maximum diameter of about 15mm by the end of the second week. This is much smaller than the lesions induced by Yaba virus infection, which reach maximum diameters of 25-45 mm. TPV lesions are benign and do not take on the appearance of the tumorous masses seen in Yaba virus disease. There is no
pustulation and only a single lesion is usually seen. Lesions occur primarily on the upper arm, face, neck, and trunk (Downie et al., 1971). Laboratory investigations of the lesions show hypertrophy and thickening of the epithelial layers of the skin with swelling and ballooning of the deeper epithelial cells (McNulty et al., 1968, Downie et al., 1971). There is little involvement in the underlying dermis as seen in Yaba virus infection (Bearcroft and Jamieson, 1958). Epithelial cells show vacuolation of cell nuclei and cytoplasmic inclusion bodies (Downie et al., 1971). In Yaba virus infection, no nuclear vacuolation has been reported. TPV infection, unlike vaccinia and variola viruses, shows little inflammatory cell infiltration of the epithelium.

The incubation period in natural human cases is unknown (Jezek et al., 1985) but the disease lasts from 3 to 4 days with even the most severely affected patients recovering without complications (Joklik, 1988b). Skin lesions disappear within 6 to 7 weeks. Clinical diagnosis of TPV is made on the basis of the characteristic skin lesions and is confirmed based on the brick-shaped morphology of the poxvirus under electron microscopy.

TPV appears to be a zoonosis, but neither the reservoir host nor the mode of transmission to man is known. Sera collected from several species of monkeys from various parts of the world including, Malaysia, India, South America, and Africa, show the presence of neutralizing antibodies to TPV only in those animals from Africa and Malaysia (Downie, 1974). As with Yaba virus (Bearcroft and Jamieson, 1958, Niven et al., 1961, Ambrus et al., 1969), a biting arthropod vector has been suggested which may possibly spread the virus from monkeys to man (Downie et al., 1971, Manson-Bahr and Downie, 1973).
Growth Kinetics

Viral adsorption assays demonstrate that 76% of TPV adsorbs to OMK cell monolayers one hour post inoculation at 10 plaque forming units (pfu)/cell (Mediratta, masters thesis, 1997). Using plaque assays, the single step growth curve for TPV was determined by Mediratta and Essani (1999). The eclipse period for TPV, which is the time from adsorption until the first appearance of intracellular infectious viral progeny (Joklik, 1966), was shown to last for 24 h.p.i at a m.o.i. of 10 pfu/cell. The average infectious virus recovered during the eclipse period was 0.18% and 0.008%. The latent phase, or the time from adsorption until the appearance of first extracellular infectious mature virion, was determined to last until 36 h.p.i. at 10 pfu/cell. At 10 pfu/cell, maximal intracellular and extracellular viral titers were seen at 96 h.p.i. The yield of virus per cell at 10 pfu/cell is 17. One important feature of poxviral multiplication is that most mature viral progeny is retained within the host cell. This was confirmed in experiments done by Mediratta and Essani (1999), where it was found that at 96 h.p.i., approximately 78% of the mature progeny was retained intracellularly and only 18% of the total viral progeny was present in the supernatant. In addition, the yield of TPV per cell obtained at 10 pfu/cell was found to be 18 times lower than for vaccinia virus (Mediratta and Essani, 1999).

Anti-Cytokine Activity in Supernatant From TPV Infected cells

Electrophoretic examination of supernatant from TPV infected cells revealed the presence of a single 38 kDa protein that was not found in mock or non-infected cell supernatant (Essani et al., 1994). This 38 kDa protein was determined to be an
early polypeptide expressed prior to viral DNA synthesis. Based on $^3$H-N-acetylglucosamine labeling, the protein was shown to be glycosylated. Binding studies using concentrated supernatant from TPV infected cells revealed that the protein bound to human (h)IFN-γ, hIL-2, and hIL-5 but not to hIL-1, hIL-8, or hIL-10. Using an assay based on $^3$H-thymidine uptake, the protein was shown to biologically inactivate hIL-2 and IL-5. Supernatant from mock infected cells did not effect thymidine incorporation. The inactivation of IFN-γ was further demonstrated by measuring the reduction in plaque formation in infected cell monolayers. This was one of the first virally encoded proteins shown to have multiple anti-cytokine activity binding and neutralizing 3 important cytokines.

Conclusions

To date there is little known about TPV. The viral genome encodes many genes, both essential and non-essential, which could potentially unlock many answers to the immune response and virus evasion. Since TPV is similar to smallpox virus but with greatly reduced virulence, it provides an ideal model to study virus-host interactions.
MATERIALS AND METHODS

Cells and Virus

Owl monkey kidney (OMK) cells (American Type Culture Collection, Rockville, MD) were grown in modified Earle's medium (MEM) supplemented with 10% (v/v) newborn calf serum (NBCS) (Life Technologies, Grand Island, NY), 2mM L-glutamine, and antibiotics (100units/ml penicillin G sodium, 100µg/ml streptomycin sulfate, 0.25µg/ml amphotericin B) (Life Technologies) [growth medium]. Cells were maintained in MEM supplemented with 2% (v/v) NBCS, 2mM L-glutamine, and antibiotics (maintenance medium). Primary human umbilical vein endothelial cells (HUVEC) (Clonetics, Walkersville, MD) were cultured in endothelial cell basal medium (EBM) supplemented with human (h) epidermal growth factor (10µg/ml), hydrocortisone (1mg/ml), GA-1000 (50mg/ml gentamicin sulfate and 50µg/ml amphotericin-B), bovine brain extract, and 2% (v/v) fetal bovine serum (FBS) (Clonetics). TPV (American Type Culture Collection) was cultivated at 37°C in OMK cell monolayers. Infected cells were maintained in maintenance medium and incubated for 10-14 days at 37°C with 5% CO₂. Infected cells were harvested and concentrated 100-fold initially by centrifugation (Damon IEC PR-600 centrifuge) at 500 x g for 30 minutes followed by ultra-centrifugation (Beckman...
Optima XL-100K ultracentrifuge; Ti-45 fixed angle rotor) at 85,000 x g for 1 hour. The pellet was resuspended in maintenance medium to a final concentration of 100X and quantitated by plaque assay (Essani, 1982). Briefly, OMK cell monolayers grown in 6-well plates were infected with 200µl each of serial ten-fold dilutions of 100X TPV. Each dilution was plated in duplicate. Virus was adsorbed at room temperature for 1 hour on a rocker table. Following adsorption, 2.5ml of overlay medium [maintenance medium with 0.5% methyl cellulose (4000 centipoises) (Fisher Scientific, Pittsburgh, PA)] was added to each well and plates incubated at 37°C with 5% CO₂ for 7-10 days or until viral plaques were visible. Medium was aspirated carefully and 1ml of 0.1% crystal violet and 10% formaldehyde in deionized water was added to each well to fix and stain the cell monolayers for 30 minutes. Plates were then rinsed and allowed to dry. Plaques were quantified to determine pfu/ml.

Virus Purification

Twenty-five 150cm² flasks, of TPV infected OMK cell monolayers, were scraped and pelleted at 85,000 x g for 1 hour. The cell pellet was resuspended in 30ml of 10mM Tris-HCl (pH 8.0) and placed in a dounce homogenizer. The suspension was adjusted to 10µg/ml deoxyribonuclease I (Boehringer Mannheim, Indianapolis, IN) and 10U/ml ribonuclease A (Sigma Biosciences, St. Louis, MO). The mixture was homogenized with 15 strokes, incubated for 20 minutes in a 37°C water bath, and dounced another 15 strokes. The virus preparation was further homogenized with 15 strokes after adjusting to 0.5mg/ml of trypsin, 10mM EDTA. Following a 15 minute incubation in a 37°C water bath, the mixture was homogenized with 10 additional
strokes. The final virus preparation was loaded onto a 40% sucrose cushion and centrifuged at 30,000 x g for 97 minutes to remove cellular debris. The resulting virus pellet was resuspended in 2ml 10mM Tris-HCl (pH 8.0). The viral suspension was incubated for 30 minutes at 37°C after adjusting to 50µg/ml of trypsin. The virus preparation was homogenized with a final 10 strokes and the virus mixture then loaded, through a 13-gauge needle, onto a freshly prepared 20-40% sucrose gradient to separate enveloped and non-enveloped virus particles. The purified virus bands were collected and centrifuged at 30,000 x g for 1 hour. The virus pellet was resuspended in 500µl of 10mM Tris-HCl (pH 8.0) and stored at -20°C.

Supernatant Preparation

Confluent OMK cells grown in 60mm dishes were infected with TPV (50 pfu/cell) and adsorbed for 1 hour at room temperature. Maintenance medium was added and cells were incubated for 6 hours at 37°C. At 6 hours-post infection, cell monolayers were washed 3X with serum-free maintenance medium. Cells were further incubated for 18 hours at 37°C. Supernatant from TPV infected cells was collected, initially centrifuged for 30 minutes (500 x g, Damon, IEC PR-6000 centrifuge), followed by 1 hour centrifugation at 85,000 x g, and then dialyzed against deionized water at 4°C. When necessary the supernatant was lyophilized, reconstituted with maintenance medium, and filter sterilized with a 0.22µm filter (Gelman Sciences, Ann Arbor, MI). Mock supernatant was collected from uninfected OMK cells and prepared in an identical manner. When necessary, cells were
radiolabeled with 50µCi/ml $^{35}$S]methionine (1142Ci/mmol from ICN Pharmaceuticals, Inc., Costa Mesa, CA) 2-18 hours post-infection.

Preparation and Analysis of DNA

Preparation of TPV Genomic DNA

TPV genomic DNA was isolated by the proteinase K/sodium dodecyl sulfate (SDS)/phenol extraction method (Ausubel et al., 1992). Briefly, purified virus was mixed with 1% SDS, 200µg/ml proteinase K (Life Technologies), 3mM mercaptoethanol and then incubated for 48 hours in a 37°C water bath. Proteinase K was added in increments over the incubation period to a final concentration of 200µg/ml. Following incubation, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, the sample mixed gently, and then centrifuged for 5 minutes in a Brinkman 5415C centrifuge (14,000 x g). The aqueous layer was collected and the procedure was repeated twice. To the recovered aqueous layer, 1/10 volume of 3M sodium acetate, pH 5.2 was added and mixed gently. Two volumes of 100% ethanol was added, mixed, and DNA precipitated at -20°C for 30 minutes. Resulting DNA was pelleted by centrifugation (14,000 x g) and washed in 70% ethanol. The DNA pellet was air dried and then in suspended 50 µl in sterile deionized water.
Restriction Analysis of DNA

Restriction endonucleases and their appropriate buffers were obtained from Genosys Biotechnologies, Inc., (The Woodlands, TX) or Life Technologies. Digestions, using 5U of enzyme per 1µg of DNA, were performed according to manufacturer’s specifications for each enzyme. The reaction volumes varied according to DNA concentrations. All reactions were incubated at 37°C for 12-18 hours. Reactions were terminated by the addition of 1X stop solution (0.5M EDTA, 65% sucrose, 0.2% bromophenol blue, and 0.2% xilenecyanol). For the generation of blunt-ended DNA, Smal restriction endonuclease was used under the following conditions: 5U of Smal (20mM Tris-HCl, pH 7.5, 1mM Na₂EDTA, 7mM 2-mercaptoethanol, 500µg/ml BSA, 50% (v/v) glycerol) per µg DNA, 1X REact 4 (20mM Tris-HCl, pH 7.4, 5mM MgCl₂, 50mM KCl) in a total volume of 10µ1 with sterile deionized water.

Dephosphorylation of pUC19

Dephosphorylation of pUC19 was accomplished using calf intestinal alkaline phosphatase (CIAP)(Life Technologies). Briefly, 1µl of CIAP (27U/µl) (25mMTris-HCl, pH 7.6, 1mM MgCl₂, 0.1mM ZnCl₂, 50% (v/v) glycerol) was added to 3-5µg of DNA along with 1µl 10X CIAP dephosphorylation buffer (50mM Tris-HCl, pH 8.5, 0.1mM EDTA) and brought up to 10µl with deionized water. The mixture was incubated at 37°C for 15 minutes then at 56°C for an additional 15 minutes. An
additional 1µl of CIAP was added and the dephosphorylation reaction reincubated as stated above. The reaction was terminated by heating at 75°C for 10 minutes.

**S1 Nuclease Digestion of Hairpins**

To clone the TPV *PstI* terminal genomic DNA fragments, the covalently linked terminal ends (hairpins) had to be cleaved. *PstI* digested fragments of TPV DNA were treated with S1 nuclease (Life Technologies) following manufacturer's directions. Briefly, 5µg of *PstI* digested TPV DNA was mixed with 1µl S1 nuclease (99.1 U/µl) (20mM Tris-HCl, pH 7.5, 0.1mM zinc acetate, 50mM NaCl, 50% (v/v) glycerol), 1µl 10X S1 nuclease buffer (300mM sodium acetate, pH 4.6, 10mM zinc acetate, 50% (v/v) glycerol), 0.83µl 3M NaCl and brought up to 10µl using deionized water and incubated at 37°C for 30 minutes. The reaction was terminated by adding 0.5µl of 0.5M EDTA (25mM). DNA was extracted twice with an equal volume of phenol:chloroform (1:1), ethanol precipitated, and reconstituted in 5µl of deionized water.

**Repair of S1 Digested Ends**

The S1 nuclease cleaved TPV DNA ends were repaired with DNA polymerase I, large (Klenow) fragment (Life Technologies). S1 nuclease cleaved TPV DNA (5µg) was mixed with 1µl 10X React 2 (500mM Tris-HCl, pH 8.0, 100mM MgCl₂, 500mM NaCl), 0.5µl DNA polymerase I, large (Klenow) fragment (10U) (50mM potassium phosphate, pH 7.0, 100mM KCl, 1mM dithiothreitol (DTT),
50% (v/v) glycerol) and deionized water to bring total volume to 10µl. The mixture was incubated at 15°C for 1 hour and the reaction terminated with the addition of 0.5µl of 0.5M EDTA (25mM).

Blunt-End Ligation

Dephosphorylated, Smal cut pUC19 was desalted and concentrated using the QIAEX II gel extraction kit (Qiagen Inc, Valencia, CA) following manufacturer’s suggestions. Cleaned, dephosphorylated Smal cut pUC19 and blunt-ended PstI digested TPV DNA were ligated using a DNA ligation kit (Sigma Biosciences). In an Epindorf tube, 1 µl 10X ligation buffer (250mM Tris-HCl, pH 7.8, 100mM MgCl₂, 10mM DTT), 60fmol (53ng) vector (Smal cut, dephosphorylated pUC 19), 180fmol (306ng) insert (blunt-ended TPV DNA fragments), 1µl T4 ligase (1U)(10mM Tris-HCl, pH 7.5, 50mM KCl, 1mM DTT, 50% (v/v) glycerol), 12% PEG, and nuclease-free water to bring volume up to 10µl were mixed gently and incubated at 14°C for 14-18 hours. The reaction was terminated by heating to 65°C for 10 minutes.

Transformation

Transformation of DH5α-MCR competent cells (Life Technologies) was performed by adding 5µl of ligation mixture to 20µl of competent cells and incubating at 4°C for 30 minutes. The mixture was then heat shocked for 45 seconds in a 42°C water bath. The tubes were removed from the water bath and then returned to 4°C for 2 minutes. SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl,
2.5mM KCl, 10mM MgCl₂·6H₂O, and 20mM glucose) was added to the mixture and incubated in a 37°C shaker for 1 hour at 225rpm. The suspension was then plated out in triplicate, using 20µl, 50µl, and 100µl of the suspension, on LB agar plates (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 10g/L dextrose, and 15g/L agarose) containing 50µg/ml of ampicillin. The plates were incubated at 37°C overnight.

Isolation of Plasmid DNA

Plasmid DNA was prepared according to the alkaline lysis method (modified from Ausebel et al., 1992). Bacterial clones were grown in LB broth (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, and 10g/L dextrose) containing 50µg/ml of ampicillin and incubated overnight in a 37°C shaker incubator (200rpm). Cells were pelleted by centrifugation at 700 x g (Damon IEC PR-6000 centrifuge), washed in 1X PBS, and repelleted at the same speed. Cell pellets were suspended in lysis buffer (25mM Tris-HCl pH 8.0, 10mM EDTA, and 50mM glucose) and transferred to 1.5ml microcentrifuge tubes. Lysis buffer with lysozyme (10mg/ml) (Sigma Biosciences) was added to disrupt bacterial cell walls and incubated on ice for 5 minutes. Alkali-SDS (0.2N NaOH and 1% SDS) was added, tubes inverted 10X, and further incubated on ice for 5 minutes. 3M sodium acetate (400µl) was added to neutralize the alkali solution and to precipitate proteins and large DNA. Cells were centrifuged at high speed (10,000 x g), incubated on ice for 15 minutes and then recentrifuged. The supernatant was removed, isopropanol added and then placed at -70°C for 30 minutes to precipitate plasmid DNA. Plasmid DNA was pelleted at 4°C by
centrifugation (10,000 x g), and resuspended in deionized water. Proteins were separated by phenol-chloroform extraction and the aqueous layer collected. To precipitate RNA, 200µl ammonium acetate (7M) was added to the aqueous layer, incubated on ice for 10 minutes, and RNA pelleted by centrifugation (10,000 x g). The supernatant was collected, 100% ethanol added, and incubated on dry ice for 10 minutes to precipitate plasmids. Plasmid DNA was pelleted and resuspended in 0.3M sodium acetate. Ethanol (100%) was once again added to precipitate plasmid and incubated on dry ice for 10 minutes. The plasmid DNA pellet was resuspended in deionized water and stored at -20°C.

Resolution and Recovery of DNA Fragments

Agarose Gel Electrophoresis

Agarose gels (0.7%) were prepared by dissolving agarose in 1X TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA, pH 8.0) by heating to a 100°C. Ethidium bromide (1.0µg/ml) was added directly to the agarose solution and the gel was then poured and allowed to solidify. DNA samples were prepared with 10X loading buffer (25% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol). Samples were electrophoresed at 50 V for 2-6 hours. Electrophoresis was in 1X TBE buffer, pH 8.0. DNA markers, λ HindIII and/or φX174 HaeIII (Life Technologies) were run alongside samples to estimate molecular sizes of DNA fragments. Following electrophoresis, gels were illuminated on an UV light source (Fotodyne
Foto/Prep UV transilluminator, Hartland, WI) and photographed with a Fotodyne FCR-10 camera using a red filter and Polaroid type 667 film.

Recovery of DNA Fragments

DNA fragments were recovered from 0.7% agarose gels using the QIAEX II gel extraction kit (Qiagen, Inc.) following manufacturer's suggestions. Briefly, the DNA band was excised from the gel, incubated at 50°C for 10 minutes in 3 volumes of buffer QXI and 10µl-30µl of QIAEX II to solubilize the agarose and bind DNA to QIAEX II silica particles. The mixture was centrifuged for 30 seconds to pellet DNA bound silica beads. The pellet was washed in 80% ethanol solution twice, and DNA eluted in 20µl of sterile deionized water.

Analysis of DNA by Blotting and Hybridization

Southern Hybridization

DNA was digested with restriction endonuclease and electrophoresed through a 0.7% agarose gel containing 1 µg/ml ethidium bromide. The gel-fractionated DNA was photographed alongside a ruler over a gel illuminator (Fotodyne Foto/Prep UV transilluminator). The gel was denatured in 1.5M NaCl and 0.5M NaOH for 1 hour at room temperature and then neutralized for another hour in 1M Tris-HCl (pH 8.0) and 1.5M NaCl. The DNA fragments were transferred to a nylon membrane (ICN Pharmaceuticals, Inc.) using standard methods as described in Maniatis et al. (1989) modified from Southern (1975). The membrane was soaked in 6X SSC (3M NaCl,
0.3M sodium citrate, pH 7.0), allowed to air dry at room temperature and then UV-crosslinked for 1 minute or baked at 80°C for 2 hours. TPV probes were designed from the N-terminus amino acid sequence of the isolated 38 kDa TPV protein (ITLKY-YTVTLKDNGLYDGVFHY-HYN-QL) (see Table 2). TPV probes were 5'-end labeled with [γ-32P ATP] (4500Ci/mmol) (ICN Pharmaceutical, Inc.) as described by Maniatis et al. (1982). Briefly, 50pmol of DNA was mixed with 20U T4 polynucleotide kinase (50% glycerol (v/v), 50mM Tris-HCl, pH 7.6, 25mM KCl, 5mM DTT, 0.1µM ATP, 0.2mg/ml BSA) (Life Technologies), 1X forward reaction buffer (350mM Tris-HCl, pH 7.6, 50mM MgCl2, 500mM KCl, 5mM 2-mercaptoethanol), and 150µCi [γ-32P ATP] (4500Ci/mmol), and then incubated at 37°C for 1 hour. Probes were purified using the Nensorb 20 nucleic acid purification cartridge (NEN Life Science Products, Boston, MA) or Probe Quant G-50 microcolumns (Amersham Pharmacia Biotech, Piscataway, NJ) as directed by manufacturer. Nensorb 20 cartridges were pre-equilibrated with reagent A (0.1M Tris-HCl, 10mM triethylamine, 1mM disodium EDTA, pH 7.7). Four hundred microliters of reagent A was added to the radiolabeled probe mixture and then passed

<table>
<thead>
<tr>
<th>Probe</th>
<th>Size</th>
<th>Tm</th>
<th>Probe Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPV-3</td>
<td>18mer</td>
<td>47°C</td>
<td>5'-TAT-GAT-GGT-GTT-TTT-TAT-3'</td>
</tr>
<tr>
<td>TPV-5</td>
<td>24mer</td>
<td>57.6°C</td>
<td>5'-GGA AAG TGT AAA GAC AAC GAA TAC-3'</td>
</tr>
<tr>
<td>TPV-6</td>
<td>24mer</td>
<td>59.3°C</td>
<td>5'-GCT-TGT-CTA-AGT-TGT-AAC-GGA-AGA-3'</td>
</tr>
<tr>
<td>MT2</td>
<td>1.1 kbp</td>
<td></td>
<td>(Upton et al., 1991)</td>
</tr>
</tbody>
</table>
through the column. The column was washed with 3ml reagent A and 3ml deionized water, respectively. The probe was eluted with reagent B (50% methanol in water), at a flow rate of 1 drop/second, and counts detected on a scintillation counter to determine fractions containing radiolabeled nucleic acid. Probe Quant G-50 microcolumns were prepared by initially vortexing to resuspend the resin bed and then pre-spinning the column for 60 seconds at 735 x g. The radiolabeled probe mixture was slowly added to the column and then centrifuged at 735 x g for 2 minutes. The purified sample was collected and counts detected on a scintillation counter.

The pMTN-6 vector (Schreiber and McFadden, 1994) containing the MT2 gene (Upton et al., 1991) was kindly supplied by Xiao-Ming Xu. The MT2 insert was excised from the pMTN-6 vector using restriction endonucleases, HindIII and PstI. The 1.1 kbp fragment was gel purified as described previously and radiolabeled with [α-32P dCTP] using the random prime DNA labeling system (Life Technologies). Briefly, 25ng of DNA was dissolved in 10µl of distilled water, denatured by boiling for 5 minutes and then immediately placed on ice. To the DNA, 15µl random prime buffer mixture (0.67M HEPES, 0.17M Tris-HCl, 17mM MgCl2, 33mM 2-mercaptoethanol, 1.33mg/ml BSA, 18(OD260)units/ml oligodeoxyribonucleotide primers (hexamers), pH 6.8), 5µl (50µCi) [α-32P dCTP] (3000Ci/mmol), 2µl of each dATP, dGTP, and dTTP solution (0.5mM), 1µl (3U) DNA polymerase, large fragment (Klenow fragment) (50mM potassium phosphate buffer (pH 7.0), 100mM KCl, 1mM DTT, 50% (v/v) glycerol) and distilled water to
49µl was added on ice. The mixture was incubated at room temperature for 1-3 hours and 5µl stop solution (0.5M EDTA, pH 8.0) was added.

Varied conditions were used for hybridization depending on the probe (see Table 3). Method 1 was utilized for TPV-probe 3 (Genosys Biotechnologies, Inc.), method 2 for TPV-probe 5 (MWG Biotech, High Point, NC), method 3 for TPV-probe 5 (MWG Biotech), and method 4 for the MT2 probe. Pre-hybridization solution was composed of 6X SSC (0.3M NaCl, 0.03M sodium citrate, pH 7.0), 5X Denhardt's solution [1% each BSA, Ficoll, PVP (Sigma Biosciences)], 0.5% SDS, and 0.1mg/ml salmon testes DNA (Sigma Biosciences). Washed blots were placed on film and stored at -80°C. Films of blots hybridized with TPV-probes were developed after 1 week. Films of MT2 probed blots were developed after 15 hours, because of higher counts detected by the Geiger counter, and the blots washed further at increasing temperatures ranging from 35°C up to 68°C.

Table 3
Hybridization Conditions

<table>
<thead>
<tr>
<th>Method</th>
<th>Pre-hybridization</th>
<th>Hybridization</th>
<th>Wash conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30°C, 4 hours</td>
<td>30°C, overnight</td>
<td>4 washes in 2X SSC and 0.1% SDS at 35°C, 15 minutes/wash</td>
</tr>
<tr>
<td>2</td>
<td>45°C, overnight</td>
<td>45°C, overnight</td>
<td>4 washes in 2X SSC and 0.1% SDS at 30°C, 15 minutes/wash</td>
</tr>
<tr>
<td>3</td>
<td>30°C, 2 hours</td>
<td>30°C, 24 hours</td>
<td>4 washes in 2X SSC and 0.1% SDS at 25°C, 15 minutes/wash</td>
</tr>
<tr>
<td>4</td>
<td>25°C, 1 hour</td>
<td>25°C, overnight</td>
<td>4 washes in 2X SSC and 0.1% SDS at 25°C-68°C, 15 minutes/wash</td>
</tr>
</tbody>
</table>
Polymerase Chain Reaction (PCR)

PCR was carried out to amplify smaller regions from the 5.0 kbp TPV PstI-J fragment for sequencing. All reagents were obtained from Life Technologies. PCR products, ranging from 600 bp to 2 kbp, were generated from 0.5µg plasmid DNA using 1.75U Taq DNA polymerase, 1X PCR buffer (20mM Tris-HCL, pH 8.4, 50mM KCl), 1.5mM MgCl₂, 0.2mM dNTPs, and 25pM each primer in a 50µl reaction. Thirty cycles of PCR amplification were performed as follows: denaturation 94°C for 30 seconds, annealing 45°C for 1 minute, extension 68°C for 2 minutes. Amplified products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

PCR products were dried down in a speed vacuum and resuspended in 10µl of water. To separate nonspecifically amplified primers, unincorporated primers, or remaining plasmid DNA, PCR products were electrophoresed on a 0.7% agarose gel in TBE buffer. DNA fragments were extracted using the QIAEX II gel extraction kit (Qiagen, Inc.) as described previously.

Cycle Sequencing and Dye Terminator Removal

Samples were cycle-sequenced using an ABI Prism big dye terminator cycle sequencing ready kit (Perkin Elmer, Foster City, CA). Template DNA (60ng), 3pmol primer, 3µl premix buffer, were mixed to a total reaction volume of 20µl.
Unincorporated dye terminators were removed using Centri-Sep columns (Princeton Separations, Inc., Adelphia, NJ) as directed by manufacture. Briefly, the matrix was prepared by hydrating the column with water, air bubbles removed, and the column was allowed to settle for 30 minutes. Interstitial fluid was drained from the column by gravitational flow, and then any remaining traces of fluid removed by centrifugation at 750 x g for 2 minutes. Sample was carefully added to the center of the column matrix, centrifuged at 750 x g for 2 minutes, and DNA collected.

**Dideoxy Sequencing Method**

Sequencing reaction products were analyzed using an ABI Prism 310 DNA sequencer (Perkin Elmer) or sent out to Biosynthesis (Lewisville, TX) for analysis. The nucleotide sequence for both strands was determined. The universal M13/pUC forward (5'-CGC-CAG-GGT-TTT-CCC-AGT-CAC-GAC-3') and reverse primers (5'-AGC-GGA-TAA-CAA-TTT-CAC-ACA-GGA-3') were used to obtain the initial sequence. From this, custom 18- to 24-mer oligodeoxyribonucleotides were synthesized using standard chemistries (Biosynthesis and Life Technologies) to primer walk the remaining fragment.

**DNA Sequence Analysis**

The Auto Assembler DNA Sequence Assembly software, version 2.0 (PE Applied Biosystems, Foster, CA) was utilized to assemble and edit DNA sequences. Searches for DNA sequence homology were performed using the BLASTX (Altschul *et al.*, 1990) program available at the National Center for Biotechnology Information
(NCBI) website (www.ncbi.nlm.nih.gov). The ORF FINDER program at the NCBI web site (www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to determine probable ORFs. Multiple alignment and comparison of DNA and protein sequences was achieved using the Sequence Navigator software (PE Applied Biosystems).

Preparation and Analysis of RNA

Preparation of Cytoplasmic RNA

The supernatant from either mock infected cells or TPV infected cells was mixed with TNF-α (50 units/ml), incubated for 1 hour at 4°C, and then added to HUVEC monolayers. The cell monolayers were incubated for 4 hours at 37°C. Total cellular RNA was extracted using acid guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). Briefly, Trizol (Life Technologies) was added to cell monolayers and placed on a shaker for 10 minutes. One hundred microliters of chloroform per 1ml of Trizol reagent was added, samples vortexed vigorously, and then incubated on ice for 15 minutes. The aqueous phase was collected and isopropanol added to precipitate the RNA. The samples were centrifuged at high speed (14,000 x g) for 20 minutes at 4°C. One ml of 70% ethanol was added to wash the RNA pellet and samples were recentrifuged at high speed for 10 minutes at room temperature. The pellets were dried in a 37°C incubator for 10 minutes and suspended in 1mM EDTA in RNase-free water.
Northern Blot Analysis

RNA (5µg) was electrophoresed through 1% formaldehyde-agarose gel containing 1µg/ml ethidium bromide. The gel-fractionated RNA was transferred to nitrocellulose membranes (Southern, 1975) and immobilized by UV irradiation. The blots were pre-hybridized for 2 hours in Rapid-hyb buffer (Amersham Int., Arlington Heights, IL) and hybridized overnight at 65°C with [α³²-P dATP] (3000Ci/mmol) (Amersham Int.) radiolabeled cDNA probes specific for ICAM-1, E-selectin, VCAM-1, or G3PDH. The blots were washed in 2X SSC (0.3M NaCl, 0.03M sodium citrate, pH 7.0) containing 0.5% SDS for 15 minutes, followed by washes in 0.4X SSC with 0.1% SDS for 30 minutes, and 0.5X SSC for 1-2 minutes respectively. The washed blots were placed on film at -80°C. ³²P-labeled DNA probes were essentially generated by random primer oligonucleotide procedure as described (Feinberg and Vogelstein, 1983). The ICAM-1 probe was an EcoR I fragment of human cDNA (Staunton et al., 1988), the E-selectin probe was a 1.53kb EcoR I fragment of human cDNA (Bevilacqua et al., 1989), and the VCAM-1 probe was a Hind III-Xho I fragment of the human cDNA consisting of nucleotides 132-1814 (Osborn et al., 1989).

DNA-Protein Interactions

Preparation of Nuclear and Cytoplasmic Extracts

Cells were washed quickly with 1ml of trypsin and then removed with the further addition of 2ml of trypsin left on for 3-5 minutes. Cells were collected,
centrifuged, suspended in buffer A (10mM HEPES, pH 7.9, 1.5mM MgCl₂, 10mM KCl, 0.1% NP-40, and 0.5mM DTT). After recentrifugation, the cells were resuspended in 80µl buffer A containing 0.1% Triton X-100 by gently pipetting. After incubation at 4°C for 10 minutes, the homogenate was centrifuged and the nuclear pellet washed in 40µl of buffer C (20mM HEPES, pH 7.9, 0.42M NaCl, 25% (v/v) glycerol, 1.5mM MgCl₂, and 0.2mM EDTA). This suspension was incubated on ice for 30 minutes and then centrifuged at 20,000 x g for 20 minutes. The resulting supernatant was stored at -70°C as nuclear extract.

**Gel Mobility Shift Assay**

The supernatant from either mock infected cells or TPV infected cells was mixed with TNF-α (50 units/ml), incubated for 1 hour at 4°C, and then added to HUVEC monolayers. Cells were harvested at 20 minutes following stimulation, and nuclear fractions prepared as described above. Total protein concentration was determined using bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) using BSA as the standard. Double-stranded (ds) oligonucleotide containing a consensus NF-κB recognition sequence (Promega, Madison, WI) was end-labeled with T4 polynucleotide kinase in the presence of [γ³²P ATP] (>7,000Ci/mmol) (Amersham Int.) (Maniatis _et al._, 1982). For the assay, 10µg of nuclear protein were incubated with 0.1pmol of [³²P] radiolabeled ds oligonucleotide in a binding buffer (12mM HEPES, 4mM Tris-HCl, 60mM KCl, 1mM DTT, 1µg poly(dI-dC), pH 7.9) for 30 minutes at room temperature (Bennett _et al._, 1996). Competition experiments were
conducted using 50-fold molar excess of the competing unlabeled ds oligonucleotide together with the labeled one. Super shift assays were conducted using antibodies to the subunits of NF-κB. Samples were loaded on a 5% 1X Tris-glycine-polyacrylamide gel (50mM Tris-Cl, 380mM glycine, 2mM EDTA, pH 8.5). Following electrophoresis the gel was dried and analyzed by autoradiography.

Analysis and Detection of Proteins

Quantitation of Proteins

Total protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) or the Lowry method (1951), using BSA as the standard.

Electrophoretic Separation of Proteins

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was used to separate proteins based on size. The lower gel was prepared with lower gel buffer (1.5M Tris-base, 0.4% SDS, pH 8.8), and 11.5% acrylamide (acrylamide:bisacrylamide ratio, 19:1). Polymerizing agents ammonium persulfate and TEMED (Life Technologies) were added immediately prior to pouring the gel. The upper gel was prepared with upper gel buffer (0.5M Tris-base, and 0.4% SDS, pH 6.8), 4.5% acrylamide (acrylamide:bisacrylamide ratio, 19:1), 30µl ammonium persulfate, and 10µl TEMED. The lower gel was first poured and allowed to polymerize for 1 hour. The upper or stacking gel was then poured on top
of the lower gel and also allowed to polymerize for 1 hour. Samples were prepared with 10X-dissociation buffer (62.5mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 3 minutes before loading. Samples were electrophoresed at 50 mA in 1X running buffer (0.025M Tris-base, 0.192M glycine, and 0.1% SDS) for 4 hours.

Staining Proteins in Gels

After electrophoresis, the gel was placed in staining solution (0.25% Coomassie brilliant blue, 50% methanol, and 10% acetic acid) at room temperature 4 hours to overnight. The gel was fixed and destained in destaining solution (5% methanol and 7.5% acetic acid) at room temperature with shaking.

Drying Gels

The destained, fixed gel was washed in deionized water, placed on a piece of Whatman 3MM paper cut to the size of the gel, and then covered on one side with plastic wrap. The gel was dried for 2 hours by vacuum and then exposed to Kodak X-Omat autoradiography film (Rochester, NY) overnight at room temperature.

Protein Purification

A TNF-α affinity column was prepared using the Aminolink Plus Coupling Gel (Pierce) and manufacturer’s specifications. Briefly, the column was equilibrated with 2.5 column volumes of pH 10 coupling buffer (0.1M sodium phosphate, 0.15M NaCl, pH 7.2), 1-2 column volumes of ligand solution (2.5mg TNF-α/3ml agarose
dialyzed against coupling buffer) was added, and the column placed on a rocker for 4 hours at 25°C to mix the reaction slurry. The column was allowed to drain and then washed with 2.5 column volumes of pH 7.2 coupling buffer (0.1M sodium citrate, 0.05M sodium carbonate). One column volume of pH 7.2 coupling buffer and 40µl of reducing agent (0.5M sodium cyanoborohydride solution) per 2ml of coupling buffer were added to the reaction mixture and then placed on a rocker for 4 hours at room temperature. BCA protein assay was used to determine the amount of ligand coupled to the gel by the difference in the amount of ligand present before and after coupling. To block any remaining active sites, the liquid coupling solution was drained and the gel was washed with 2 column volumes of quenching buffer (1M Tris-HCl, pH 7.4). Following washing, 1 column volume of the quenching buffer and 0.2 column volumes of reducing solution were added to the column and the column mixed on a rocker for 30 minutes. Following mixing, the column was drained, and first washed with at least 10 column volumes of wash solution (1M NaCl), then washed twice with 2.5 column volumes of degassed storage solution (0.05% sodium azide in PBS). The top porous disc was placed into the column with 2.5 column volume wash and pushed down to 1mm of the gel surface. Filter sterilized \[^{35}S\] labeled supernatant from TPV infected cells (preparation described above) was passed through the TNF-\(\alpha\) affinity column. The column was washed with 1X PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na\(_2\)HPO\(_4\), 1.4mM KH\(_2\)PO\(_4\), pH 7.0) until no radioactivity was detected in the flow through. The TNF-\(\alpha\) binding proteins were eluted with 1M acetic acid, dialyzed overnight at 4°C against several changes of
deionized water, and lyophilized. Eluted TNF-α binding proteins were reconstituted in deionized water and analyzed by polyacrylamide gel electrophoresis.

**Protein Sequence Analysis**

Supernatant from TPV infected cells and mock infected cells (described above) was lyophilized and reconstituted in serum-free growth medium. Samples were boiled for 3 minutes, loaded on a 10-20% Tris-Glycine pre-cast gel (10-20% acrylamide, 2.6% bisacrylamide, pH 8.6) (Novex, San Diego, CA), and run at 120 constant voltage for 2 hours in Tris-Glycine running buffer (29g/L Tris base, 144g/L glycine, pH 8.3). Low and mid-range markers (Promega) were run alongside samples for size determination. Following electrophoresis, the gel was equilibrated with two 15 minute washes in buffer C (25mM Tris, 40mM norleucine, pH 9). For each gel, 4 filters (3M from Biorad, Hercules, CA) were equilibrated in buffer C, 2 filters in buffer B (25mM Tris, pH 10.5), and 3 filters in buffer A (300mM Tris, pH 10.5). A polyvinylidene fluoride (PVDF) membrane (0.45µM, Millipore Corp. Inc., Bedford, MA) was dipped in 100% methanol for 10 seconds prior to rinsing for 10 seconds in water and then equilibrated in buffer C. The blotting sandwich for the gel was arranged from the positive to the negative electrode as follows: (a) 4 filters in buffer C, (b) PVDF membrane in buffer C, (c) gel in buffer C, (d) 2 filters in buffer B, and (e) 3 filters in buffer A. The transfer was conducted at 1.5 mA constant current per centimeter squared PVDF membrane for 55 minutes. After electroblotting, the blot was stained for visualization in Coomassie blue stain for 15 minutes and then destained in 10% acetic acid and 50% methanol. The membrane was air-dried. The
38 kDa protein band was excised from the PVDF membrane and directly sequenced using N-terminal analysis by automated Edman degradation.

**Enzyme Linked Immunosorbent Assay (ELISA)**

Prepared supernatants from TPV infected cells and mock infected cells were pre-incubated with either TNF-α (50 units/ml) or IL-1β (0.25ng/ml) for 1 hour at 4°C and then added to HUVEC monolayers in 96-well tissue culture plates. Cells were incubated for 4 hours at 37°C. Cells were then washed three times with PBS (pH 7.0), fixed with 1% paraformaldehyde in PBS for 15 minutes, and washed again in PBS, three times. Required primary antibody in PBS (1µg/ml) with 0.1% BSA was added to each well and cells further incubated for 2 hours at 37°C. Following incubation, cells were again washed and incubated for 2 hours with alkaline phosphatase-conjugated goat anti-mouse IgG (Cappel, West Chester, PA) in PBS with 0.1% BSA. To detect the binding of secondary antibody, p-nitrophenylphosphate (pNPP) alkaline phosphatase substrate (1mg/ml) (Sigma Biosciences) in diethanolamine solution (1M diethanolamine, 0.5mM MgCl₂, 0.02g/dl NaN₃, pH 9.8) was added. The plates were incubated for 20-40 minutes at 37°C and then read on an ELISA microplate reader at an OD of 405nm.
RESULTS

Inhibition of CAM Gene Expression

In the inflammatory response, recruitment of leukocytes to sites of injury and infection is mediated by the expression of adhesion molecule receptors on the surface of endothelial cells. Specific cell adhesion molecules (CAMs) such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), or E-selectin are expressed on endothelial cells in response to inflammatory stimuli (Pober and Cotran, 1991). Cytokines such as TNF-α, IFN-γ, and IL-1β are known to up-regulate the expression of CAMs. Soluble poxviral homologs of cytokine receptors have been identified to be secreted from virus infected cells (Shchelkunov et al., 1993a, Buller and Palumbo, 1991, Smith and Goodwin, 1995, Smith, 1994), which inhibit the activity of various cytokines crucial to the inflammatory response including TNF-α and IFN-γ. Knowing the anti-cytokine activity of supernatant from TPV infected cells (Essani et al., 1994), the present study was undertaken to examine the effects that this supernatant from TPV infected cells could have on the inflammatory response. As endothelial cells are closely associated with inflammation, these cells could be used to examine the effects of supernatant from TPV infected cells on CAM gene expression. Total RNA was prepared from human umbilical vein endothelial cells (HUVEC) treated with supernatant from TPV infected cells pre-incubated with TNF-α. Treatment of HUVEC with TNF-α
normally induces the expression of E-selectin, ICAM-1, and VCAM-1 genes (Figure 6, lane 2). The cellular extracts were run on a 1% formaldehyde-agarose gel. Northern blot analyses were performed to measure CAM gene expression. As shown in Figure 6, RNA isolated from cells treated with supernatant from mock infected cells showed that TNF-α induced CAM gene expression was unaffected (Figure 6, lane 3). Levels of expression were equivalent to those of cells treated with TNF-α alone (Figure 6, lane 2). These results indicate that mock supernatant is not interfering with TNF-α induced CAM gene expression. However, cells treated with supernatant from TPV infected cells (Figure 6, lane 4) showed complete inhibition of TNF-α induced CAM gene expression. These results suggest that supernatant from TPV infected cells is interfering with the transcription of TNF-α induced CAM genes.

Selective Down-Regulation of TNF-α Induced CAMs

To explore whether TNF-α induced CAM down-regulation through TPV infected cell supernatant was selective to TNF-α, HUVEC were exposed to IL-1β (0.25ng/ml), also an inducer of CAM expression. HUVEC were stimulated for 4 hours with supernatant samples prepared as described previously. Using quantitative ELISA to measure CAM expression, we found that supernatant from TPV infected cells down-regulated TNF-α induced expression of E-selectin (Figure 7B), ICAM-1 (Figure 7D), and VCAM-1 (Figure 7E). Down-regulation of ICAM-1, E-selectin, and VCAM-1 were comparable to non-stimulatory levels. This was not seen with mock
Figure 6. Inhibition of CAM Gene Expression by Supernatant From TPV Infected Cells. Total RNA was isolated and electrophoresed through 1% formaldehyde-agarose gel. The fractionated RNA was transferred to nitrocellulose membrane and hybridized to either [\textsuperscript{32}P]-labeled human (a) E-selectin-specific, (b) ICAM-1-specific, or (c) VCAM-1-specific cDNA and analyzed by autoradiography. Lane 1, untreated; lane 2, TNF-\(\alpha\) treated; lane 3, supernatant from mock infected cells mixed with TNF-\(\alpha\) (50 units/ml); and lane 4, supernatant from TPV infected cells mixed with TNF-\(\alpha\) (50 units/ml), respectively. (d) G3PDH, a constitutive housekeeping gene, was used as an indicator of mRNA loading. All experiments were repeated at least three times.
Figure 7. Selective Down-Regulation of CAMs by Supernatant From TPV Infected Cells. Primary human endothelial cells were stimulated for 4 hours with supernatant from either mock infected cells or TPV infected cells, pre-incubated for 1 hour with either IL-1β (A and C) or TNF-α (B, D, and E). Quantitative ELISA was carried out to monitor the expression of selectin (A and B), ICAM-1 (C and D) or VCAM-1 (E). Values on the vertical axis represent the means ± SE (sd) for triplicate samples. Numbers on the horizontal axis represent volumes of supernatant from mock infected cells shown by gray bars and supernatant from TPV infected cells shown on the right by black bars.
infected cell supernatant. In contrast, IL-1β induced CAM expression was unaffected by supernatant from either mock infected or TPV infected cells (Figure 7A, 7C). These results indicate a selective down-regulation of only TNF-α induced CAM expression by supernatant from TPV infected cells.

**Inhibition of NF-κB Activation and Translocation**

To further explore the nature of inhibition, we investigated the nuclear transcription factor-kappa B (NF-κB) regulated pathway of CAM gene expression. NF-κB is a two subunit complex involved in the regulation of numerous genes, including those involved in the inflammatory response. NF-κB is bound to an inhibitory molecule, IκB, in the cytoplasm of unstimulated cells. Addition of pro-inflammatory cytokines such as TNF-α and IL-1β activates a signal transduction cascade, which leads to the phosphorylation of IκB and the subsequent degradation of this inhibitory molecule. The loss of IκB activates NF-κB, resulting in its translocation to the nucleus where it initiates transcription of CAM genes (reviewed in Baeuerle, 1991). To determine whether the supernatant from TPV infected cells was interfering with the NF-κB pathway, we employed gel electrophoretic mobility shift assays (GEMSAs). GEMSAs would allow us to ascertain activation of NF-κB by measuring NF-κB DNA binding in nuclear extracts (Figure 8). Unstimulated cells (Figure 8, lane 1) do not show NF-κB DNA binding activity. However, when these cells are treated with TNF-α (Figure 8, lane 2), the DNA binding activity of NF-κB
Figure 8. Inhibition of NF-κB DNA Binding Activity by Supernatant From TPV Infected Cells. Confluent HUVEC were either untreated (lane 1), treated with TNF-α (lane 2), treated with supernatant from mock infected cells with TNF-α (lane 5), or supernatant from TPV infected cells with TNF-α (lane 6). Nuclear extracts were assayed for NF-κB DNA binding activity by gel mobility shift assays using radiolabeled oligonucleotide containing a consensus NF-κB recognition sequence. NF-κB DNA binding can be completely inhibited using 50 fold molar excess of unlabeled oligonucleotide (lane 3) and super-shifted with antibodies to the NF-κB subunits (lane 4).
can be seen, as would be expected. Nuclear extracts of cells that were treated with supernatant from mock infected cells, pre-incubated with TNF-α (Figure 8, lane 5), displayed NF-κB DNA binding activity to the same degree as extracts from cells treated with TNF-α alone. Supernatant from mock infected cells did not interfere with the DNA binding ability of NF-κB. However, extracts from cells that were treated with supernatant from TPV infected cells, pre-incubated with TNF-α (Figure 8, lane 6) showed no NF-κB DNA binding activity. These results indicate that active NF-κB is not found in nuclear extracts from cells treated with the supernatant from TPV infected cells. This suggests a complete inhibition of NF-κB activation and translocation by the supernatant from TPV infected cells.

TPV Infected Cells Secrete a 38 kDa Polypeptide That Binds to TNF-α

Taken together, these results show that supernatant from TPV infected cells selectively inhibits TNF-α induced CAM expression prior to NF-κB activation. This then suggests that inhibition is at the receptor binding level with a constituent of the supernatant directly binding to TNF-α and inhibiting it from inducing CAM expression. To address this possibility and to identify this component of the supernatant, [35S]methionine labeled supernatant from TPV infected cells was examined electrophoretically on an 11.5% polyacrylamide gel (Figure 9A). Supernatant from TPV infected cells (Figure 9A, lane 2) shows a single polypeptide, approximately 38 kDa that is not found in supernatant from mock infected cells (Figure 9A, lane 1). To determine if this polypeptide was binding to TNF-α, a TNF-
Figure 9. TPV Infected OMK Cells Secrete a 38 kDa Polypeptide That Binds TNF-α. Infected OMK cells were labeled with [35S]methionine 2-18 hours post infection as described under Materials and Methods. (A) Radiolabeled supernatant was loaded on an 11.5% polyacrylamide gel. Electrophoretic examination of supernatant from mock infected cells (lane 1) and supernatant from TPV infected cells (lane 2) are shown. (B) Supernatant from TPV infected cells was run through a TNF-α affinity column. The column was then washed, TNF-α binding proteins eluted, and each eluted fraction was analyzed on an 11.5% polyacrylamide gel (lanes 1-6).
α affinity column was prepared. [35S]methionine labeled supernatant from TPV infected cells was run through the column, and non-binding proteins removed with multiple PBS washings. TNF-α binding proteins were then eluted with 1M acetic acid, and each eluted fraction was analyzed on an 11.5% polyacrylamide gel. Autoradiograms revealed one major 38 kDa protein band (Figure 9B). This band is also the same molecular weight as a TPV polypeptide previously described by our lab (Essani et al., 1994). These results clearly indicate that a 38 kDa protein found in the supernatant of TPV infected cells binds directly with TNF-α and suggests that this activity is responsible for the inhibition of CAM expression in TNF-α treated cells.

Identification of the 38 kDa Protein

To identify the TPV 38 kDa protein, concentrated supernatant from TPV infected cells was prepared and loaded on a 10-20% polyacrylamide Tris-Glycine gel. Following electrophoresis, proteins were transferred to a PVDF membrane by electroblotting, and then stained with Coomassie blue for visualization. The 38 kDa protein band, as determined by low and mid-range markers (Figure 10, lanes 5 and 6), was excised and directly sequenced using N-terminal analysis by automated Edman degradation. Sequence analysis provided the amino acid sequence, Ile-Thr-Leu-Lys-Tyr-X-Tyr-Thr-Val-Thr-Leu-Lys-Asp-Asn-Gly-Leu-Tyr-Asp-Gly-Val-Phe-Tyr-X-His-Tyr-Asn. The amino acid sequence was compared to other proteins using the program BLASTN (Altschul et al., 1990). Exhaustive search of the protein databank did not reveal any homology between the 38 kDa TPV protein and any known protein.
Figure 10. TPV Infected Cells Secrete a 38 kDa Protein. Concentrated supernatant from TPV infected cells was prepared as described and loaded on a 10-20% polyacrylamide Tris-Glycine gel (lanes 5 and 6). Following electrophoresis, proteins were transferred to a PVDF membrane by electroblotting, and then stained with Coomassie blue for visualization. The 38 kDa protein band, as determined by low-mid-range and low-high range markers (lanes 1-2 respectively), was excised and directly sequenced using N-terminal analysis by automated Edman degradation. This 38 kDa was not seen in supernatant collected from mock infected cells (lanes 3 and 4).
Cloning and Sequencing of the Gene Encoding the 38 kDa Protein

To isolate the open reading frame (ORF) encoding the 38 kDa TPV protein, cloned TPV PstI DNA fragments inserted into the pUC 19 vector (PstI fragments F, H, J, L, M, and N) and genomic TPV DNA were digested with PstI and electrophoresed on a 0.7% agarose gel containing ethidium bromide (1.0µg/ml) (Figure 11). Genomic DNA from OMK cells was run as a control to confirm that the 38 kDa protein was viral and not cellular in origin. The DNA was transferred by Southern blotting and then probed with TPV probe-3 (5'-TAT-GAT-GGT-GTT-TTT-TAT-3'). TPV-probe 3 was designed from the N-terminal amino acid sequence of the 38 kDa protein. Of the fractionated DNA, only one showed clear and distinct hybridization to the probe (Figure 11). This band was identified as the 5.1 kbp PstI-J fragment. The PstI-J fragment exists between the 10.1 kbp PstI-D fragment and the 7.9 kbp PstI-G fragment.

The TPV PstI-J Fragment

Both strands of the entire 5.1 kbp cloned J fragment were sequenced to identify the ORF encoding the 38 kDa protein. The universal M13/pUC forward (5'-CGC-CAG-GGT-TTT-CCC-AGT-CAC-GAC-3') and reverse primers (5'-AGC-GGA-TAA-CAA-TTT-CAC-ACA-GGA-3') were used to obtain the initial sequence. From this, custom 18- to 24-mer oligodeoxyribonucleotide primers were synthesized using standard chemistries to primer walk the remaining fragment. A sequencing scheme is shown in Figure 12. The auto assembler DNA sequence assembly
Figure 11. TPV Probe-3 Binds to TPV PstI-J Fragment. TPV PstI digested cloned fragments F (9.0 kbp), H (6.6 kbp), J (5.1 kbp), L (2.1 kbp), M (1.4 kbp), and N (1.1 kbp) were run on a 0.7% agarose gel. Fractionated DNA was transferred to a nylon membrane and hybridized with $\gamma^{32}$P ATP labeled TPV Probe-3 (see tables 2 and 3). Figure shows photograph of ethidium bromide stained agarose gel (B) and corresponding autoradiogram (A).
software was utilized to assemble and edit DNA sequence data. The 5.1 kbp sequence of the J fragment is displayed in Figure 13. Nucleotide sequence analysis of the 5.1 kbp PstI-J fragment using the NCBI ORF finder revealed 7 potential ORFs (Figure 14). DNA sequence data did not reveal any amino acids with homology to the 38 kDa protein. Five of the ORFs however did have direct counterparts in the vaccinia virus. Table 4 lists the ORFs with their respective sizes and homologies.

The TPV PstI-J1R ORF is predicted to encode a protein of 385 amino acids with 69% homology to the vaccinia virus, Copenhagen strain H4L ORF found on the HindIII H fragment of the genome (Rosel et al., 1986). The vaccinia virus H4L ORF encodes the RNA polymerase-associated transcription specificity factor, RAP 94. The TPV PstI-J1R ORF is most likely the partial sequence of a larger gene, which continues on the TPV

![Sequencing Scheme for the TPV PstI 5.1kbp J Genomic Fragment.](image)

Both strands of the 5, 137 bp were sequenced. The universal M13/pUC forward and reverse were used to obtain the initial sequence. From this, custom 18- to 24-mer primers were synthesized using standard chemistries to primer walk the remaining fragment. The TPV PstI 5.1 kbp J genomic fragment is shown in the 5' to 3' direction. TPV-J fragment (TJ) up (3') primers are designated with a U, and down (5') primers are designated with a D.
Figure 13. DNA sequence of the 5.1 kbp PstI-J fragment of TPV Genome. The sequence is shown in the 5' to 3' direction. Both strands of DNA were sequenced.
Figure 13. continued from previous page.
Figure 13. continued from previous page.
Figure 13. continued from previous page.
Figure 14.  *PstI* Restriction Map of the TPV Genomic DNA and Location of the Potential ORFs on the *PstI* J Fragment.  (A) The *PstI* restriction map of the TPV genome is shown with fragments A through N designated according to their sizes.  (B) The potential ORFs on the J fragment are shown with respect to the 5', 3' sequence of the J fragment.
## Table 4
Putative Open Reading Frames of the TPV PstI-J Fragment

<table>
<thead>
<tr>
<th>ORF</th>
<th>Size (Bases)</th>
<th>MW (kDa)</th>
<th>Homologies</th>
</tr>
</thead>
</table>
| J1R  | 1158         | 42.9     | 91% to vaccinia H4L homolog of Yaba virus  
69% to vaccinia H4L protein-encodes RNA polymerase-associated transcription specificity factor (RAP94) |
| J2L  | 123          | 4.6      | no significant homology found                                                                                                                                                                    |
| J3L  | 165          | 6.1      | 37% to feline sarcoma virus tyrosine kinase transforming protein  
37% to human macrophage colony stimulating factor I receptor precursor (CSF-1)  
37% to feline sarcoma virus CSF-1 receptor oncogene homolog |
| J4R  | 972          | 36.0     | 80% to vaccinia H3L homolog of Yaba virus  
51% to P32 antigen of sheeppox virus  
35% to vaccinia immunodominant envelope protein P35 |
| J5L  | 573          | 21.2     | 84% to vaccinia H2R of homolog of Yaba virus  
62% to vaccinia virus H2R-late protein  
26% to human T-cell receptor α-chain precursor V-region |
| J6R  | 516          | 19.1     | 88% to vaccinia H1L homolog of Yaba virus  
63% to vaccinia protein-tyrosine phosphatase (late protein H1)  
29% to human dual specificity protein phosphatase 7 |
| J7L  | 1464         | 54.2     | 91% to vaccinia J6R homolog of Yaba virus  
78% to RNA polymerase subunit rpo 147 of vaccinia virus  
79% to myxoma virus m68R |
sheeppox virus H4L homolog, 72% to the myxoma virus H4L homolog (m72L), 73% to the rabbit fibroma virus H4L homolog (gp072L), 68% to variola virus H4L homolog (ORF5L), 58% to the molluscum contagiosum subtype 1 H4L homolog (mc085L), 48% to the orf virus H4L homolog (F2L), and 22% to the *Melanoplus sanguinipes* entomopoxvirus H4L homolog (msvl19).

The TPV *Pstl-J2L* ORF is predicted to encode a 40 amino acid protein, which has no significant homology to any known proteins. The TPV *Pstl-J3L* ORF is predicted to encode a 54 amino acid protein which shows 37% homology to the transforming protein of the McDonough strain of feline sarcoma virus (FSV) and 37% homology to the human macrophage colony stimulating factor-1 (CSF-1) receptor precursor. The carboxyl terminus of the FSV transforming protein interestingly has homology to the tyrosine-specific protein kinases encoded by many known retrovirus oncogenes (Hampe et al., 1984).

The TPV *Pstl-J4R* ORF is predicted to encode a protein composed of 323 amino acids. This protein shows 80% homology to the vaccinia virus H3L homolog of the Yaba virus, 52% homology to the P32 antigen of the lumpy skin disease virus, 51% homology to the sheeppox virus P32 antigen, 35% homology to the IMV membrane associated protein encoded by the H3L ORF of the vaccinia virus, and 35% homology to the H3L protein of the variola virus. The TPV *Pstl-J4R* ORF contains a translation initiation site, TAAATGG, at the start of the ORF and therefore most likely encodes a late protein.

The TPV *Pstl-J5L* ORF is predicted to encode a 190 amino acid protein, which shows significant homology to the vaccinia virus H2R ORF (62%). There is
84% homology to the H2R homolog of the Yaba virus, 64% to the H2R homolog of the sheeppox virus, 63% to the myxoma virus H2R homolog, 61% to the rabbit fibroma virus H2R homolog, and 52% to the molluscum contagiosum virus H2R homolog. The vaccinia virus WR strain H2R encodes a late protein called H2. Interestingly the TPV Psrl-J5L ORF also shares 26% homology with the human T-cell receptor-α chain precursor V-region.

The TPV Psrl-J6R is predicted to encode a 171 amino acid protein, which is the TPV protein-tyrosine phosphatase gene and shows notable homology to the vaccinia virus tyrosine phosphatase encoded on the H1L ORF. The vaccinia virus H1L protein is a soluble protein with dual tyrosine and serine specificity (Guan et al., 1991). This protein is essential to the virus and allows it to dephosphorylate phosphotyrosyl and phosphoseryl residues in tissue culture (Guan et al., 1991). The TPV Psrl-J6R also shows significant homology to other poxvirus tyrosine phosphatases (Mossman et al., 1995a) including Yaba virus (88%), myxoma virus (76%), sheeppox virus (75%), rabbit fibroma virus (75%), and variola virus (63%). The vaccinia virus protein-tyrosine phosphatase is encoded by a late gene with a late translation initiation site found upstream of the 5' end of the ORF. This TAAATGG sequence is found on the TPV Psrl-J7R ORF but at the 5' end.

The TPV Psrl-J7L ORF is the largest gene encoded on the TPV Psrl-J fragment and is located at the 3' terminal. The Psrl-J7L protein shows significant homology to the RPO147 subunit, of the vaccinia virus DNA-directed RNA polymerase (Broyle and Moss, 1986). The RNA polymerase subunit, encoded on the vaccinia virus J6R gene, is a 147 kDa polypeptide. Our Psrl-J7L ORF encodes a 487
amino acid protein (54.2 kDa) which appears to be the partial sequence of a larger one. The remaining protein sequence may be encoded on the 7.9 kbp TPV PstI-G fragment. The PstI-J7L ORF shows 91% homology to the Yaba virus J6R homolog, 70% to the myxoma virus J6R homolog (m68R), 79% to the rabbit fibroma virus J6R homolog (gp068R), and 88% to the sheeppox virus J6R homolog.

To summarize, the PstI-J fragment is 5,137 bases in length and potentially encodes 7 major ORFs. Two of these ORFs, the TPVPstI-J1R and -J7L, are partially encoded and are found at the 5' and 3' terminals respectively. Late genes, PstI-J4R and -J6R, contain the translational initiation sequences, TAAATGG as determined for vaccinia virus (Rosel et al., 1986). The TPV PstI-J7L is the largest ORF and shows 78% homology to the vaccinia virus J6R gene encoding the DNA-directed RNA polymerase 147 kDa subunit. This gene is highly conserved among other poxviruses including the myxoma virus, the sheeppox virus, the rabbit fibroma virus, and the variola virus. The TPV PstI-J6R ORF has significant homology with the vaccinia virus H1L encoding tyrosine phosphatase. There is 80-91% sequence homology between Yaba virus and TPV.
DISCUSSION

Much of the information on poxviruses stems from extensive studies on the vaccinia virus, which has become the prototype of the poxviruses. The viruses included in the *Yatapoxvirus* genus, however, are relatively uncharacterized in comparison to the vaccinia virus. The members of this genus, Yaba virus, Yaba-like disease virus (YLDV), and TPV are three distinct viruses. DNA restriction maps illustrate the extent of differences between the Yaba virus, YLDV, and TPV (Knight et al., 1989). All three viruses included in the genus, *Yatapoxvirus* have been reported to be immunologically related (Niven *et al.*, 1961, Downie *et al.*, 1971).

The serological similarities between YLDV and TPV and the relatedness of clinical disease they produce in man and monkey led to the early misidentification of the two viruses as one (Downie and Espana, 1973). This continues to cause ambiguity in the nomenclature between YLDV and TPV. ATCC retains stocks of virus which it identifies as TPV although restriction endonuclease analysis in our laboratory shows that the virus is actually YLDV. Exhaustive search of the early literature reveals that TPV was originally isolated from human skin lesions and that Yaba virus and YLDV were originally isolated from monkey lesions (Downie *et al.*, 1970, Bearcroft and Jamieson, 1958, Espana, 1966 Hall and McNulty, 1967, Crandell *et al.*, 1969, Espana, 1971). Although Knight *et al.* (1989) recognize YLDV as the causative agent of disease in monkeys and TPV as the causative agent of human
disease, it is now clear that both viruses cause identical disease in humans and monkeys (Hall and McNulty, 1967, Crandell et al., 1969, Downie, 1974).

Initial work conducted in our laboratory demonstrated that supernatant from TPV infected cells possessed multiple anti-cytokine activities. This was demonstrated using cytokine (IL-2, IL-5, IFN-γ) binding and inhibition assays (Essani et al., 1994). This activity was not seen in supernatant from uninfected cells. SDS-PAGE analysis confirmed the presence of a single 38 kDa protein in the supernatant from TPV infected cell that was not detected in the supernatant from uninfected cells. This protein was, therefore, postulated to cause the inhibition of all three cytokines. Knowing the anti-cytokine potential of the supernatant from TPV-infected cells, we were interested to investigate the effects of the supernatant on cell adhesion molecule (CAM) gene expression and NF-κB regulation. This dissertation reports the selective inhibition of TNF-α induced CAM expression and the inhibition of NF-κB activation mediated by a 38 kDa protein secreted from TPV-infected cells and not uninfected cells.

In order to survive, animal viruses have evolved mechanisms to interfere with the potentially lethal host inflammatory response. TNF-α is one of the central mediators in the inflammatory response. TNF inactivation enables the virus to evade this host anti-viral defense. The results presented here demonstrate that supernatant from TPV-infected cells contains an activity capable of selectively down-regulating the expression of TNF-α induced, but not IL-1β induced, endothelial CAMs (Paulose et al., 1998). CAM expression appears associated with the presence of a TNF-α
binding, 38 kDa protein found only in the supernatant from TPV-infected cells. Other poxviruses have been shown to encode proteins with homology to the TNF-α receptor, for example the Shope fibroma virus T2 protein (Smith et al., 1991), the myxoma virus MT-2 protein (Upton et al., 1991), and the CrmB and CrmC proteins of the cowpox virus (Hu et al., 1994). The results presented here provide evidence that the 38 kDa protein found in the supernatant from TPV infected cells binds directly to TNF-α and inhibits it from activating the NF-κB regulated CAM pathway. It appears that this may be the same protein from TPV that has previously been shown to bind to hIL-2, hIL-5, and IFN-γ (Essani et al., 1994). The ability of a single viral polypeptide to bind multiple host cytokines illustrates the evolutionary and genetic efficiency of the virus in its attempts to overcome the host anti-viral response. The concept of multi-functional viral proteins however is not limited to the protein described here. The parainfluenza virus (Kingsbury, 1990), the Kaposi's sarcoma-associated herpesvirus (Kledal et al., 1997), the adenovirus (Hoffman et al., 1992), the myxoma virus (Macen et al., 1996, Lalani et al., 1997), and the cowpox virus (Tewari and Dixit, 1995) also express proteins with multiple biological activities. The CrmA protein of the cowpox virus, for example, inhibits both interleukin-1β production and cytokine-induced apoptosis (Tewari and Dixit, 1995). The myxoma virus MT7 protein binds to IFN-γ (Upton et al., 1992) and in addition also interacts with members of the chemokine superfamily involved in the inflammatory response (Lalani et al., 1997). The MT2 protein of the myxoma virus binds and inhibits TNF
and also blocks apoptosis by a mechanism that is independent of TNF (Upton et al., 1991, Macen et al., 1996).

This multiple cytokine binding property does raise questions concerning the mechanism(s) involved in the binding. One possibility is that hTNF-α, hIL-2, hIL-5, and hIFN-γ share a common binding site. Alternatively, there may be an accessory protein (either cellular or viral) that acts as a bridge between the cytokine and the 38 kDa protein. To address this question and to further characterize the TPV 38 kDa protein, attempts were made to identify and isolate the gene encoding it.

The TPV 38 kDa protein was isolated and subsequently 25 amino acid residues were sequenced from the unblocked N-terminus. This sequenced peptide showed no homology to any known proteins. Multiple TPV-oligonucleotide probes were designed from the 25 amino acid sequence (Table 3). In the numerous blots and hybridizations that were carried out, only TPV-probe 3 hybridized to the genomic TPV DNA. Figure 11 illustrates these results. TPV-probe 3 hybridized to the PstI-J fragment of the TPV genome. TPV-probe 3 failed to hybridize to any of the other fractionated genomic TPV DNA fragments. The PstI-J fragment was isolated and both strands of the entire 5.0 kbp fragment completely sequenced. DNA sequence data did not reveal any amino acids with homology to the 38 kDa polypeptide.

However, there were 15 bases that were 100% identical to the TPV-probe 3 sequence, which may have resulted in the specific binding of the probe. Multiple attempts were made to isolate the ORF encoding the 38 kDa protein, but none were successful. The degeneracy of the genetic code, the sequences of the probes, and the low stringency of hybridizations probably contributed to the failure. However, sequence analyses of
the TPV PstI-J fragment reveals 7 potential ORFs with significant homology to the Yaba virus. The PstI-J7L ORF encodes the partial sequence of the TPV DNA-directed RNA polymerase. There is significant conservation between the TPV PstI-J7L gene and other poxviral RNA polymerase genes. The PstI-J6R ORF shows notable homology to the vaccinia virus tyrosine phosphatase gene. The gene encoding the tyrosine phosphatase was shown to be an essential gene in the myxoma virus and the Shope fibroma virus possessing dual specificity to tyrosine and serine (Mossman et al., 1995a). The TPV PstI-J2L ORF shows homology to the transforming protein of the feline sarcoma virus which has homology to the tyrosine specific protein kinase encoded by many known retroviral oncogenes (Hampe et al., 1984). The TPV PstI-J2L gene may be an additional virulence factor encoded by TPV. The potential ORFs found on the TPV PstI-J fragment are located near one another and many times the adjacent strand ORFs overlap. This is similar to the gene organization described for vaccinia virus (Rosel et al., 1986, Traktman, 1990a).

Poxviruses are unique among animal viruses. Their genomes are large and inclusive ranging from 130 kbp in the parapoxvirus (Menna et al., 1979, Robinson et al., 1987) to approximately 300 kbp in avipoxviruses (Hyde et al., 1967). This enables the poxviruses the ability to encode a vast array of proteins needed for replication, transcription, and virulence. Poxviruses have evolved to acquire one of the most impressive arrays of genes encoding proteins for immune evasion. Many of these genes encode homologs of host immune components. In the past ten years, there has been a significant advancement in our understanding of these virally encoded immunomodulating viral proteins. These viral homologs contain a multitude
of information on the immune response and viral infection. They may in turn lead to new strategies for novel anti-viral and anti-inflammatory therapeutics.
Appendix A

Biosafety Clearance
January 24, 2000

TO WHOM IT MAY CONCERN:

This is to certify that Mini Paulose-Murphy has not used animals in studies included in this thesis. All experiments were carried out in a BSL-2 laboratory in this department, and she was certified to use radioactive materials.

Biosafety Clearance

Karim Essani, Ph.D.
Professor
Appendix B

Radiation Safety
This certifies that [Name] has completed a 10-hour radiation safety training course at Western Michigan University.

I am hereby designated and responsible for the radiation safety training information that I have received as it pertains to the ALARA (as low as reasonably achievable) radiation exposure practices for supervision and/or use of radioactive materials and/or radiation machines. I agree to follow the rules and regulations of the Western Michigan University Radiation Safety Program, State of Michigan's "Ionizing Radiation Rules," Michigan Department of Consumer & Industry Services and Department of Environmental Quality rules and regulations, and the U.S. Nuclear Regulatory Commission's licensing specifications. Further, I have read and understand the information that has been given to me at the Radiation Safety Training course as it pertains to the use of USNRC Licensed Material.

This certificate expires on: 11/24/99

I agree to attend a refresher radiation safety training course prior to the date of expiration in order to continue to use radiation machines or radioactive materials at W.M.U.

Date of Birth: 12/13/70 Social Security #: 075-56-2891
BIBLIOGRAPHY


but not all mouse cell lines against lysis by tumor necrosis factor. J. Virol. 65: 4114-4123.


Schreiber, M., Rajarathnam, K., and McFadden, G. (1996). Myxoma virus T2 protein, a TNF receptor homolog, is secreted as a monomer and dimer that each bind rabbit TNFα but the dimer is a more potent TNF inhibitor. J. Biol. Chem. 271: 1333-13341.


