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MOLECULAR CHARACTERIZATION OF POXVIRAL RING FINGER PROTEINS: VIROSOME LOCALIZATION AND IDENTIFICATION OF DNA BINDING AND APOPTOSIS INHIBITION ACTIVITY

by

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B.Sc., National University of Ireland, Galway, 1994

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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in the Department of Biochemistry and Microbiology

We accept this dissertation as conforming

to the required standard

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ABSTRACT

Shope fibroma virus (SFV) N1R is a member of a family of poxvirus proteins that is associated with virulence and largely defined by the presence of a C-terminal RING finger motif and localization to virus factories within the cytoplasm of infected cells. Altered proteins, with deletions and site-specific mutations, were transiently expressed in vaccinia virus infected cells to discern regions of the protein that are required for localization. Deletion mutagenesis implicated a requirement of a small central region of the RING for localization, but the RING motif alone was not sufficient. A chimeric protein, however, in which the RING motif of the herpes simplex virus-1 ICP0 protein replaced the SFV N1R RING motif did localize to virus factories, indicating that the specificity for factory localization resided outside the RING motif of N1R. Critical evaluation of an alignment of poxviral N1R homologs identified a short, highly conserved N-terminal sequence 24-YINIT-28. When this sequence was deleted from N1R localization was abolished.

Recombinant N1R protein isolated from vaccinia virus (VV) infected cells bound to calf-thymus DNA cellulose. Elution from this matrix required 0.5-0.75M NaCl, suggesting N1R localizes to the factory through an inherent DNA binding activity. Structural prediction analysis inferred that the conserved N-terminal region required for N1Rs factory localization forms a short β strand and subsequent alignment analysis with β sheet DNA binding proteins uncovered significant homology with the ribbon-helix-helix motif family which utilize a short β sheet for specific DNA interaction. Characterization of the factory localization of five N1R mutants, each having a single potential β strand residue replaced with Ala revealed that Asn 26 was the most important residue for factory localization.

In contrast to N1R, which strongly binds DNA and rapidly sediments with the virus factories, SFV-N1RAsn26ΔAla mutant protein was found in the soluble fraction of infected cell lysates and failed to bind DNA cellulose. These results indicate that the N1R RING

finger motif may not be central to DNA interactions and that N1R β strand residues particularly Asn 26 are involved in DNA binding and targeting N1R to the virus factories.

Overexpression of N1R in vaccinia virus (VV) infected cells was found to inhibit virus induced apoptosis. To clarify the role of N1R protein with respect to apoptosis and to examine whether the related ectromelia virus virulence factor p28 (EVp28) might also play a role in apoptosis protection, a p28- mutant EV virus and the VV-N1R virus were tested for their ability to interfere with apoptosis induced by different signals.

VV and EV infection were found to protect cells from Ultra Violet (UV) light, Tumor necrosis factor alpha (TNFα) and anti-Fas induced apoptosis. Expression of SFV N1R and EVp28 however, only protected infected HeLa cells from apoptosis induced by UV light, and did not protect from apoptosis induced by TNFα or anti-Fas antibody. Immunoblot analysis indicated EVp28 blocks processing of procaspase-3 suggesting EVp28 acts upstream of this protease in response to UV induced apoptotic signals. The requirement of EVp28 to promote replication and virulence *in vivo* may be related to apoptosis suppression because the number of progeny virus harvested from p28- mutant EV virus infected cells compared to wild type EV was similar following mock UV induced apoptosis, but significantly reduced following apoptosis induction by UV.

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ABBREVIATIONS USED

α,	alpha
2-5A,	2-5-linked oligoadenylate
aa,	amino acid
Abs,	absorbance
AIDS,	acquired immunodeficiency syndrome
AIF,	apoptosis inducing factor
ANT,	adenine nucleotide translocator
Apaf-1,	apoptosis protease activating factor-1
Asn,	asparagine
ATM,	ataxia telangiectasis mutated
ATP,	adenosine triphosphate
Atr,	AT and rad-related
β,	beta
BARD,	BRCA1-associated RING domain 1
BGMK,	baby green monkey kidney
BRCA,	breast cancer susceptibility gene
BrdU,	bromodeoxyuridine
BSC-1,	African green monkey cells
C-terminus,	carboxyl terminus
CaPV,	canarypox virus
caspase,	cysteinyl aspartate-specific proteinase
CHX,	cycloheximide
CMV,	cytomegalovirus
CPV,	cowpox virus
CR1,	complement factor receptor 1
Cys,	cysteine
DAPI,	4',6'-diamidino-2-phenylindole
DED,	death effector domain
DEPC,	diethyl pyrocarbonate
DISC,	death inducing signaling complex
DMF,	N, N dimethyl formamide
D-MEM,	Dulbecco's modified Eagle medium
DNA,	deoxyribonucleic acid
DNA-PK,	DNA activated protein kinase
DNase,	deoxyribonuclease

ds, double stranded
ECL, enhanced chemiluminescence
EDTA, ethylene diamine tetraacetic acid
EGTA, ethylene bis(oxyethylenenitrilo)-tetraacetic acid
EGF, epidermal growth factor
eIF2, eukaryotic translation initiation factor 2
ELISA, enzyme-linked immunosorbent assay
EPV, entomopoxvirus
ER, endoplasmic reticulum
EV, ectromelia virus
FADD, FAS-associated death domain
FasL, Fas ligand
FCA, flow cytometric analysis
FITC, fluorescein-isothiocyanate
FLIPs, FLICE inhibitory proteins
FPV, fowlpox virus
γ, gamma
Gly, glycine
gpt, xanthine-guanine phosphoribosyltransferase
HHV1, human herpes virus type 1
His, histidine
HIV, human immunodeficiency virus
HSV, herpes simplex virus
IAP, inhibitor of apoptosis
ICAD, inhibitor of caspase-activated DNase
ICE, interleukin-1β converting enzyme
IEEHV, immediate early equine herpes virus protein
IFN, interferon
IGF-1, insulin-like growth factor-1
IGIF, interferon-g-inducing factor
IkB, inhibitor of kappa B
IL, interleukin
Ile, isoleucine
IMP, inflammatory modulatory protein
IPTG, Isopropyl-1-thio-β-D-galactosidase
k, kappa

LB, Luria broth
Leu, leucine
Lys, lysine
mAb, monoclonal antibody
MAC, membrane attack complex
MCV, molluscum contagiousum virus
MCS, multiple cloning site
MDM2, murine double minute clone 2
Mel18, melanoma 18 protein
MHC, major histocompatibility complex
MOI, multiplicity of infection
MPV, monkeypox virus
MsEPV, *Melanoplus sanguinipes* entomopoxvirus
MYX, myxoma virus
N-terminus, amino terminus
NF-kB, nuclear factor kappa B
NIK, NFkB inducing kinase
NK, natural killer
NMR, nuclear magnetic resonance
OD, optical density
ORF, open reading frame
PARP, poly (ADP-ribose) polymerase
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PDGF, platelet-derived growth factor
PEG, polyethylene glycol
pfu, plaque forming units
Phe, phenylalanine
PI, propidium iodide
PKR, protein kinase p68
PML, promyelocytic leukemia protein
PMSF, phenylmethylsulfonyl fluoride
PT, permeability transition
PVDF, polyvinylidene difluoride
RAG1, recombination activating gene 1
Rb, retinoblastoma protein

RBQ-1, retinoblastoma binding protein Q1
RFLP, restriction fragment length polymorphism
RING, really interesting new gene
RNA, ribonucleic acid
RNase, ribonuclease
RPV, rabbitpox virus
RT, room temperature
SCID, severe combined immunodeficiency
SD, standard deviation
SDS, sodium dodecyl sulphate
SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM, standard error of the mean
SFV, Shope fibroma virus
SPV, swinepox virus
TB, Terrific broth
TFIIIA, transcription factor IIIA
TK, thymidine kinase
TNF, tumor necrosis factor
TNFR, tumor necrosis factor receptor
TRADD, TNFR-associated death domain
TRAF, tumor necrosis factor receptor associated factor
TRAIL, TNF-related apoptosis-inducing ligand
TRAMP, TNF-receptor-related apoptosis-mediated protein
Tris, tris(hydroxymethyl)aminomethane
ss, single stranded
SSC, standard saline citrate
US, United States
UV, ultra-violet
VAR, variola virus
vCCI, viral CC-chemokine inhibitor
VCKBP, viral chemokine binding protein
VDAC, voltage dependent anion channel
VGF, vaccinia growth factor
VV, vaccinia virus
WHO, World Health Organization
X-gal, 5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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David

I've walked these streets
a virtual stage
it seemed to me
make up on their faces
actors took their
places next to me

I've walked these streets
in a carnival
of sights to see
all the cheap thrill seekers
the vendors and the dealers
they crowded around me

have I been blind
have I been lost
inside my self and
my own mind
hypnotized
mesmerized
by what my eyes have seen?

I've walked these streets
in a spectacle of wealth and poverty
in the diamond markets
the scarlet welcome carpet
that they just rolled out for me

I've walked these streets
in the mad house asylum
they can be
where a wild eyed misfit prophet
on a traffic island stopped
and he raved of saving me

have I been blind
have I been lost
inside my self and
my own mind
hypnotized
mesmerized
by what my eyes have seen?

have I been wrong
have I been wise
to shut my eyes
and play along
hypnotized
paralyzed
by what my eyes have found
by what my eyes have seen
what they have seen

'*Carnival*' by Natalie Merchant.

GENERAL INTRODUCTION

The *Poxviridae* comprise a family of large DNA viruses that are characterized by a number of features: a large brick-shaped or ovoid virions, a genome consisting of a single linear molecule of covalently closed, double stranded DNA between 130 and 300 kb in length and notably among DNA viruses, viral replication exclusively within the cytoplasm of the infected cell (Moss, 1996b). Poxviruses are a group of highly successful pathogens. Smallpox, caused by the orthopoxvirus variola virus (VAR), was once the most serious disease of humankind and claimed millions of lives (Fenner, 2000). It was the first human disease to be eradicated globally, the result of a 10 year vaccination program effort instigated by the World Health Organization (WHO) (Fenner, 2000). The recent emergence of monkeypox virus (MPV) infections of humans, with symptoms similar to smallpox, in Africa has once again drawn attention to these viruses (Cohen, 1997). Furthermore, the increased incidence of Acquired Immunodeficiency Syndrome (AIDS) has resulted in a significant number of severe infections by the opportunistic tumorigenic poxvirus, molluscum contagiosum (MCV) (Senkevich *et al.*, 1996).

Interest in poxviruses stems from their use as model systems to study viral genes, their specific roles in neutralizing host defenses and also as vector systems for delivery of genes of therapeutic interest (Moss, 1996a; Paoletti, 1996). As a group, poxviruses infect a wide range of hosts (Upton *et al.*, 1994), and give rise to either a localized or generalized infection, typically characterized by a prominent vesicular rash (Buller and Palumbo, 1991).

Classification

The *Poxviridae*, as a family, are ubiquitous, infecting mammals, birds, reptiles, and invertebrates. The *Poxviridae* family is divided into two subfamilies: *Chordopoxvirinae* (poxviruses of the vertebrates) and *Entomopoxvirinae* (poxviruses of the insects) (Moss, 1996b). Viruses of the subfamily *Chordopoxvirinae* are subdivided into eight genera: Orthopoxviruses; Parapoxviruses; Capripoxviruses; Suipoxviruses; Leporipoxviruses; Avipoxviruses; Yatapoxviruses and Molluscipoxviruses which are distinguished from each other primarily by serologic cross-reaction and cross-protection as well as host range (Fenner, 2000; Moss, 1996b). Viruses of the subfamily *Entomopoxvirinae* are similarly subdivided into three genera based primarily on differences in viral host range and virion morphology. Genus A viruses infect coleopterans, genus B viruses infect lepidopterans and orthopterans, and genus C viruses infect dipterans (Afonso *et al.*, 1999).

Natural History of Poxviruses

Chordopoxvirinae

(1) Orthopoxviruses

Orthopoxviruses are undoubtedly the best studied poxvirus genus and include variola virus, vaccinia virus, cowpox virus, monkeypox virus and ectromelia virus. The most notorious member of this family is variola virus, the causative agent of smallpox. For thousands of years, this virus was responsible for a devastating disease of human populations with high case fatality and transmission rates (Henderson, 1999). Smallpox is believed to have appeared at the time of the first agricultural settlements in northeastern Africa, around 10, 000 BC. The earliest evidence of skin lesions resembling those of small pox is found on the faces of mummies from the time of the 18th and 20th Egyptian

Dynasties (1570 to 1085 BC) and in the well preserved mummy of Pharaoh Ramses V, who died as a young man in 1157 BC. The first recorded smallpox epidemic occurred in 1350 BC during the Egyptian-Hittite war (Barquet and Domingo, 1997).

Smallpox shaped the development of western civilization. The disease is credited with destroying at least three empires. Five reigning European monarchs died from smallpox during the 18th century. The first stages of the decline of the Roman Empire, around AD 180, coincided with a large-scale epidemic: the plague of Antonine, which killed between 3.5 and 7 million people. The Arab expansion, the Crusades, and the discovery of the West Indies all contributed to the spread of the illness (Barquet and Domingo, 1997).

Unknown in the New World, smallpox was introduced by Spanish and other European explorers and colonizers. It decimated the local population and was instrumental in the fall of the empires of the Aztecs and the Incas. When the Spanish arrived in 1518, the region that is now Mexico had about 25 million inhabitants; by 1620, this number had diminished to 1.6 million. A similar decrease occurred on the eastern coast of what became the United States, where the advent of smallpox had disastrous consequences for the native populations. The disease continued to be spread through the relentless process of European colonization (Barquet and Domingo, 1997).

The symptoms of smallpox as it was known in 18th-century England appeared suddenly and included high fever, chills or rigors, cephalalgia, characteristic dorsal-lumbar pain, myalgias, and prostration. Nausea and vomiting were also common. After 2 to 4 days, the fever relented and a rash appeared on the face and inside the eyes; the rash frequently covered the whole body. These maculopapular skin lesions evolved into vesicles and pustules and finally dried into scabs that fell off after 3 or 4 weeks. Complications included blindness, pneumonia and kidney damage (Barquet and Domingo, 1997; Mayers, 1999).

Variola virus can remain viable for one year in dust and cloth (McGovern *et al.*, 1999), and the disease spread with ease from one individual to another by way of droplets from the nose and mouth, contact with the dried scabs of the pustules and even contact with clothes or articles used by people with smallpox. In addition, virus spread was aided by the time lapse of 12 -15 days between infection and appearance of disease symptoms (McGovern *et al.*, 1999). The case-fatality rate associated with smallpox varied between 1% and 40% and left most survivors with disfiguring scars (Barquet and Domingo, 1997). Variola major, the more serious form of smallpox, had a case fatality rate of 30-40% (Ellner, 1998). In contrast, variola minor was a less severe form of the disease and killed from 1-5% of those infected (Ellner, 1998). The case-fatality rate in the infant population was even higher; among children younger than 5 years of age in the 18th century, 80% of those in London and 98% of those in Berlin who developed the disease died (Barquet and Domingo, 1997).

Of those that survived smallpox, many were left blind as a result of corneal infection or scathed by unsightly scars. It was, however, recognized that those who recovered and survived from the disease were resistant to subsequent smallpox infection. This was exploited in the technique known as variolation that was introduced into Europe from the Mideast in the early 18th century. Physicians and others intentionally infected healthy persons with the smallpox virus in the hope that the resulting infection would be less severe than the naturally occurring illness and would create immunity (Barquet and Domingo, 1997), Virus and various forms of material isolated from persons with mild cases of smallpox were administered to healthy individuals in different ways. Samples from vesicles, pustules and scabs were introduced to recipients through the nose or skin. The word variola (smallpox) was used for the first time by Bishop Marius of Avenches (near Lausanne, Switzerland) in AD 570. It came from the Latin word varius, meaning "stained", or from

varus, meaning "mark on the skin". In England, the term small pockes (pocke meaning sac) was first used at the end of the 15th century to distinguish the illness from syphilis, which was then known as great pockes (Barquet and Domingo, 1997; Fenner, 2000).

The English aristocrat Lady Mary Wortley Montague was responsible for the introduction of variolation into England. She had an episode of smallpox in 1715 that disfigured her face, and her 20-year-old brother had died of the illness 18 months earlier. Lady Montague's husband, Edward Wortley Montague, was appointed British Ambassador to Turkey in 1717 and upon visitation of the Ottoman court, she observed the procedure, which was usually carried out by old women. Lady Montague was so impressed by the Turkish method that she ordered the Embassy surgeon, Charles Maitland, to inoculate her 5-year-old son in March 1718. On returning to London in April 1721, she had Maitland inoculate her 4-year-old daughter in the presence of the physicians of the Royal court. Her successful reports led to the introduction of the technique into England (Barquet and Domingo, 1997).

Even though variolation was successful, it a dangerous practice. Two to three percent of variolated persons died of smallpox, became the source of a new epidemic, or developed other illnesses from the donor's sample, such as tuberculosis or syphilis. Nonetheless, case-fatality rates were 10 times lower than those associated with naturally occurring smallpox. Variolation was a common preventative method used in China, the Middle East, and Africa well into the early parts of the 20th century (Barquet and Domingo, 1997).

In England, Edward Jenner, a country physician was experimenting with variolation when he learned from patients that milkmaids infected with a disease called cowpox were somehow protected from smallpox. Jenner had the insight to exploit this observation and performed an experiment that laid the foundation for the eradication of smallpox and

transformed humankind's fight against disease. The moment came in May 1796, when a milkmaid named Sarah Nelmes developed cowpox through contact with a cow. On 14 May 1776, Jenner extracted fluid from a pustule on her hand and used it to inoculate a healthy 8-year-old boy named James Phipps through two half-inch incisions on the surface of the arm. Six weeks later, Jenner variolated the child but produced no reaction. He performed the procedure again some months later with the same result (Moss, 1996a).

This new procedure became known as vaccination (L. *Vacca* cow) to distinguish it from the process of variolation. Although vaccination was met with initial skepticism, the success of Jenner's technique led to the rapid spread of prophylactic vaccination against infection by smallpox (Moss, 1996a). Strictly speaking, Jenner did not directly discover vaccination but he was the first person to confer scientific status on the procedure and was the instigator of its popularization (Barquet and Domingo, 1997).

Cowpox virus was later replaced by vaccinia virus, a closely related virus, which produced a milder vaccination reaction (Moss, 1996a). Despite the profound differences in human virulence of variola, vaccinia, and cowpox viruses, they are now known to be very similar and have been placed in the same orthopoxvirus genus, accounting for their ability to cross protect (Moss, 1996a). Vaccination was almost universally adopted worldwide around 1800, but it took a major commitment from the WHO in 1965 to achieve eradication of smallpox (Mayers, 1999).

Smallpox vaccination, however, is associated with some risk for adverse reactions, the two most serious being postvaccinal encephalitis and progressive vaccinia (Henderson, 1999). Post vaccinal encephalitis occurs at a rate of 3 per million primary vaccinees; 40% of the cases are fatal, and some patients are left with permanent neurological damage. Progressive vaccinia occurs among those who are immunosuppressed because of a

congenital defect, radiation therapy or AIDS. The vaccinia virus continues to replicate and unless these patients are treated with vaccinia immune globulin they may not recover (Henderson, 1999).

Ultimately the success of vaccination against smallpox culminated in the declaration in 1980 by the assembly of the World Health Organization that smallpox had been eradicated and the recommendation that smallpox vaccination be discontinued. The last reported natural infection occurred in Somalia on 26th October 1977 (Mayers, 1999). Variola virus has again recently enjoyed the scientific limelight, with the decision of the Clinton administration in April 1999, not to proceed with the planned destruction of all strains of smallpox virus presently stored in the high-security facilities at the Centers for Disease Control and Prevention in Atlanta, Georgia, and at the Institute for Viral Preparations in Moscow (Wadman, 1999). The main arguments for destruction of these stocks are that release of the virus from the laboratories would be a serious threat to human health because worldwide vaccination programs ceased in the 1970s and the availability of cloned DNA fragments of the full genome sequence of several strains of variola virus will allow most scientific questions about the properties of the viral genes and proteins to be resolved (Henderson, 1998; Wadman, 1999). However, it is naive to assume that these are the only stocks in existence worldwide and the numbing potential threat of the use of smallpox as a bioterrorist weapon by rogue nations has led to their continued preservation (Fenner, 2000) and prompts the question whether global smallpox vaccination programs should be reinstated.

Vaccinia virus

Vaccinia virus is the name given to the agent used for Jennerian vaccination. The origins of vaccinia virus are unknown (Moss, 1996b). It is not known whether vaccinia virus is the

product of genetic recombination, a new species derived from cowpox virus or variola virus by serial passage, or the living representative of a now extinct natural virus (Buller and Palumbo, 1991). Vaccinia has been isolated on occasion from outbreaks of disease in domestic animals, especially buffalo in India, but this is thought to result from contact of these animals with vaccinated humans (Buller and Palumbo, 1991).

The advent of recombinant DNA technology, together with the large size of the poxviral genome, has enabled judicious removal of undesirable poxviral genes and insertion of genes coding for immunizing antigens of a variety of pathogens (Moss, 1996a). The vaccine potential of recombinant vaccinia virus is highlighted by the development of an effective oral wildlife rabies vaccine; however, no product for use in humans has yet been licensed (Paoletti, 1996).

Cowpox virus

Although cowpox virus (CPV) was named as a result of its association with pustular lesions on the teats of cows and the hands of milkers, there is no evidence that cows act as the natural reservoir of the virus since cowpox infection is very rare in cattle. The virus is geographically distributed throughout Western Europe and wild rodents may serve as a reservoir (Buller and Palumbo, 1991). Cowpox has been described in humans, cats and other animals (Baxby *et al.*, 1994; Buller and Palumbo, 1991). Human cowpox is a rare but relatively severe zoonotic infection. Patients present with painful, haemorrhagic pustules or black eschars, usually on the hand or face, accompanied by oedema, erythema, lymphadenopathy, and systemic involvement (Baxby *et al.*, 1994). Severe, occasionally fatal, cases occur in eczematous and immunosuppressed individuals although cowpox has not yet been reported in anyone infected with the human immunodeficiency virus (HIV) (Baxby *et*

al., 1994). In Britain, several reports link cowpox infection of children with frequent close contact with domestic cats (Baxby *et al.*, 1994; Stolz *et al.*, 1996).

Monkeypox

Monkeypox virus (MPV) was discovered as a disease of laboratory primates in Copenhagen in 1958 and it caused several other outbreaks in captive primates before it was recognized as the cause of a smallpox-like disease in western Africa in 1970 (Mayers, 1999). Since then, human monkeypox has been recorded sporadically. The usual presentation is a fever lasting up to 4 days, followed by smallpox-like skin eruptions. In addition, there may be marked lymphadenopathy (Ivker, 1997). Although the mortality rate from the disease is generally low, there have been reported cases of death attributed to MPV. Most cases occur in remote villages of Central and West Africa close to tropical rainforests where there is the opportunity for contact with infected animals. MPV is usually transmitted to humans from squirrels and primates (Mukinda *et al.*, 1997). The disease is preventable by the vaccination against smallpox. The economically motivated ending of vaccination programs for smallpox has in part contributed to the reemergence of human monkeypox in the late 1990s. An outbreak in Zaire (1996-1997) represents the largest cluster of MPV cases ever reported, and the proportion of patients that were 15 years of age or older (27%) was higher than previously reported (8%) (Heymann *et al.*, 1998).

An interesting and disturbing feature of this latest outbreak is that MPV in eastern Zaire may be exhibiting inter-human transmission rates higher than seen previously during the post smallpox surveillance period suggesting that the MPV may be rapidly evolving (Chen *et al.*, 2000; Heymann *et al.*, 1998). The rate of transmission from person-to-person (73%) was higher than previously reported (30%) and this was associated with the

clustering of cases in household compounds and prolonged chains of transmission from person-to-person. Although, the proportion of deaths (3%) was lower than previously reported (10%), all age groups were affected, with unvaccinated children at the highest risk of death, about a 10% case fatality rate (Heymann *et al.*, 1998).

MPV poses a potential localized public health problem in Africa. The potential use of vaccination to protect the population at risk has inherent difficulties because the spiraling prevalence of HIV among African populations poses a high risk for the development of generalized vaccinia (Heymann *et al.*, 1998) . Currently, the WHO is monitoring the situation in Africa closely through the strengthening of detection systems for MPV and exhaustive epidemiological investigation such that future large-scale outbreaks may be avoided.

Ectromelia virus

Ectromelia virus (EV) was discovered in 1930 by Marchal as a virus infection that was naturally transmitted from one mouse to another in a research mouse colony (Fenner, 2000). EV, the agent of mousepox, has been recognized as a relatively common infection of laboratory mouse colonies in Europe, Japan and China. Disastrous outbreaks of the disease among laboratory mice in the United States, following the importation of mice from Europe in the 1950s, led to restrictions on the study of EV in the US (Fenner, 2000). However, laboratory studies have since shown that EV has a very narrow host range and infects only certain mouse species (Buller and Palumbo, 1991).

Although all laboratory mouse strains (derived from *Mus musculus domesticus*) exposed to virus become infected, some are resistant to disease (Buller and Palumbo, 1991). Susceptible mice generally die of acute hepatitis following infection, however, those that do not die of acute hepatitis develop a rash late in the infection (Fenner, 2000). The natural reservoir of EV is unknown, however, it is thought that wild mice may be involved (Buller

and Palumbo, 1991). A number of different strains of EV have been isolated which differ in their virulence for mice. The Moscow, Hampstead, and NIH 79 strains are the most thoroughly studied; the Moscow strain being the most virulent and infectious for mice (Buller and Palumbo, 1991).

(2) Parapoxviruses

Poxvirus infections are widespread in sheep, goats and cattle and can be transferred to humans through occupational exposure (Fenner, 1990). Two notable parapoxviruses of domestic animals are orf virus (synonyms: contagious pustular dermatitis, contagious ecthyma, scabby mouth), normally a disease of sheep and milker's nodule virus (synonyms: pseudocowpox, paravaccinia), normally a disease of cattle (Fenner, 1990). Human infection occurs through abrasions of the skin and localized lesions are usually found on the hands but may be transferred to the face. The lesions of orf virus are rather large painful nodules due largely to inflammation of the surrounding skin. The lesions of milker's nodule virus are highly vascularized, producing a purple colour. They are relatively painless but may itch (Fenner, 1990).

(3) Capripoxviruses

Among domestic species, capripoxvirus infections are restricted to cattle, sheep and goats. Members of this genus include sheeppox, goatpox and lumpy skin disease virus (LSDV) (Kitching, 1994). Experimentally, it is possible to infect cattle, sheep or goats with isolates derived from any of these three species. The lesions of capripoxvirus are not

restricted to the skin, but may also affect any of the internal organs, in particular the gastrointestinal tract and the respiratory tract (Kitching, 1994). Capripoxviruses of sheep and goats is enzootic in Africa, the Middle East, India, China and other parts of Asia (Kitching, 1994). In 1984, a capripoxvirus infection entered Bangladesh developing into a severe epidemic causing high mortality in the indigenous goat population (Kitching *et al.*, 1987). Capripoxviruses may be transmitted mechanically to susceptible goats by the fly (*Stomoxys calcitrans*) (Mellor *et al.*, 1987). Sheeppox was eradicated from Britain in 1866 and from other European countries in the late 1960s, however, sporadic cases have been reported (Kitching, 1994).

LSDV primarily infects cattle and often occurs in epizootic form (Davies, 1991). The disease is characterized by the eruption of nodules in the skin, which may cover the whole of the animal's body. Lesions are often found in the mouth and upper respiratory tract and systemic effects include pyrexia, anorexia, dysgalactia and pneumonia (Davies, 1991). The severity of the disease varies considerably between breeds of cattle and many suffer severe emaciation. The skin lesions cause permanent damage to the hides. The mode of transmission of the disease has not been clearly established (Davies, 1991). Contact infections do not readily occur and the evidence from the epizootiology strongly suggests that insect vectors are involved (Davies, 1991). The disease was confined to sub-Saharan Africa until recently when it appeared in epizootic form in Egypt and Israel (Yeruham *et al.*, 1995). Capripoxviruses remain largely uncharacterized at the molecular level.

(4) Suipoxviruses

Swinepox virus (SPV), the sole member of the genus Suipoxvirus, has been observed sporadically in domestic pig (*suidae sp.*) populations throughout the world (Barcena *et al.*, 2000). Congenital SPV infection has also been described; newborn pigs have lesions over their entire bodies (Borst *et al.*, 1990). SPV, however, is not considered a

serious pathogen because infected animals usually have moderate symptoms and completely recover from the infection. Although SPV is largely uncharacterized at the molecular level (Barcena and Blasco, 1998), it is a potential vector for the construction of recombinant vaccines for pigs (Tripathy, 1999) since it shows an extremely narrow host range *in vivo* and does not transmit to humans.

(5) Leporipoxviruses

Shope (or rabbit) fibroma virus (SFV) belongs to the Leporipoxvirus genus, a group of viruses that infect rabbits, hares and squirrels. SFV was originally described by Richard Shope in 1932 as an infectious agent which gave rise to fibroxanthosarcoma-like tumors in its natural host, the eastern cottontail rabbit *Sylvilagus floridanus* (McFadden, 1994). Leporipoxviruses appear to be transmitted from rabbit to rabbit by biting insects. The widespread prevalence of antibodies to the virus suggests that SFV infections may be endemic throughout North American rabbit populations (Willer *et al.*, 1999). Similar disease symptoms have been reported in the African hare *Lepus capensis*, suggesting the range of Leporipoxviruses may extend as far as African rabbit populations (Willer *et al.*, 1999). Healthy adult rabbits mount an effective cell-mediated immune response that typically starts to reduce virus lesions at 10-12 days post-infection. SFV, however, can cause a lethal disseminated infection in newborn and immunocompromised adult rabbits (Willer *et al.*, 1999).

Immunological studies and DNA sequence analysis have shown that SFV is closely related to myxoma (MYX) virus. MYX came to prominence in the 1950s when it was used as a biological agent for the control of wild rabbit populations in Europe and Australia (Fenner, 2000). MYX causes a benign infection in its evolutionary host, the North American

brush rabbit (*Sylvilagus californicus*) or the South American tapeti (*Sylvilagus brasiliensis*), but it causes a rapid systematic and lethal infection known as myxomatosis in European rabbits (*Oryctolagus cuniculus*) with mortality rates up to 100% (Cameron *et al.*, 1999). Myxomatosis is an extensively characterized veterinary disease that provides a well-defined *in vivo* model for the study of virus encoded virulence factors, including those involved in immunomodulation. The symptoms and mortality rates associated with myxomatosis are believed to be the result of multiorgan dysfunction coupled with uncontrolled secondary Gram-negative bacterial infections due to a progressive impairment of the host cellular immune response (Cameron *et al.*, 1999). MYX is transmitted mechanically via arthropod vectors, most notably the mosquito (Cameron *et al.*, 1999; Fenner, 2000).

The initial release of MYX into the Australian feral rabbit population in 1950 produced enormous moralities (Fenner, 2000), however, the effectiveness of the approach was not sustained, due to the combination of increased host resistance in the surviving rabbit populations and genetic attenuation of field virus strains (Cameron *et al.*, 1999). The genome sequence of SFV and MYX have been recently determined (Cameron *et al.*, 1999; Willer *et al.*, 1999).

(6) Avipoxviruses

Avipoxviruses are a large virus group which infect more than 60 species of wild birds representing 20 families (Afonso *et al.*, 2000). Avipoxvirus diseases of poultry and other domestic birds such as canaries and pigeons have significant economic impact worldwide, with losses resulting from a drop in egg production in layers, reduced growth rate in broilers, blindness and death (Afonso *et al.*, 2000). Fowlpox virus (FPV), the prototypical member of the Avipoxvirus genus, infects chickens and turkeys (Afonso *et al.*,

2000). Two forms of the disease are associated with different routes of infection. The most common, the cutaneous form, occurs following infection by biting arthropods that serve as vectors for mechanical viral transmission. The disease is characterized by an inflammatory process with hyperplasia of the epidermis and feather follicles, scab formation, and desquamation of the degenerated epithelium, and it additionally predisposes the host to secondary bacterial infections (Afonso *et al.*, 2000). The second, or diphtheric form, involves droplet infection of the mucous membranes of the mouth, the pharynx, the larynx and the trachea. The prognosis with this form of the disease is poor because lesions often cause death by asphyxiation (Afonso *et al.*, 2000).

Vaccination with live attenuated FPV and canarypox virus (CaPV) and nonattenuated pigeonpoxvirus is used to control this disease. Vaccination confers protective immunity 10 to 14 days after infection (Afonso *et al.*, 2000). Avipoxviruses are also of considerable interest because of their use as recombinant vaccines. Multivalent recombinant FPV vaccines, which incorporate immune response modifiers have been constructed. Recombinant FPV vaccines expressing foreign antigens have been utilized to immunize animals against other avian and mammalian diseases (Afonso *et al.*, 2000). For example, a FPV based recombinant expressing the Newcastle disease virus fusion and hemagglutinin glycoproteins has been shown to protect commercial broiler chickens for their lifetime when the vaccine was administered at 1 day of age (Paoletti, 1996).

Avipoxvirus based recombinant vaccines are attractive because of their limited host range. Although FPV and CaPV infect mammalian cells and express early viral proteins at appreciable levels, these viruses cannot complete the replication cycle in mammalian cells (Afonso *et al.*, 2000). Inoculation of avipox-based recombinants into mammalian cells has resulted in expression of the foreign gene and the successful induction of protective immunity (Paoletti, 1996). Avipox recombinants are endowed with a considerable safety profile because immunization can be affected in the absence of productive replication. This

eliminates the potential for dissemination of the vector within the vaccinate and also, the spread of the vector to nonvaccinated contacts or to the general environment (Paoletti, 1996).

(7) Yatapoxviruses

This genus is represented by Yaba virus and the prototypic Tanapox virus. Yaba disease was first observed in 1958 in an outbreak of subcutaneous tumors in captive rhesus monkeys (*Macaca mulatta*) and a dog faced baboon (*Pabio pabio*) housed in open air pens in Yaba, Nigeria (Buller and Palumbo, 1991). Spontaneous disease has been detected only in Asian monkeys (*M. mulatta; M. irus* [cynomolgus]) (Buller and Palumbo, 1991). Humans, rhesus and cynomologus monkeys appear to be the most susceptible hosts. The geographical distribution of Yaba virus remains unknown (Buller and Palumbo, 1991).

Tanapox was first recognized in 1957 in the Tana River area of Kenya (Knight *et al.*, 1989a). It is a zoonosis, with human cases having only been observed in the Tana valley and Zaire (Jezek *et al.*, 1985; Manson-Bahr and Downie, 1973). The disease is characterized by a mild febrile illness with one or two skin lesions (Essani *et al.*, 1994). The distribution, transmission and extent of human infection are largely unknown (Knight *et al.*, 1989a).

(8) Molluscipoxviruses

Molluscum contagiosum virus (MCV), a human poxvirus, is the sole member of the molluscipoxvirus genus and is related only distantly to the orthopoxviruses such as variola or vaccinia (Senkevich *et al.*, 1997). MCV has a worldwide distribution and commonly

produces 3-5 mm papules that may persist in the skin of young children and sexually active adults for months to years before spontaneously regressing (Senkevich *et al.*, 1996). In immunodeficient individuals, however, the skin lesions can become extensive, and MCV is a common disfiguring and untreatable opportunistic infection of AIDS patients (Senkevich *et al.*, 1996).

MCV infection typically elicits a weak immune response and almost no inflammatory reaction around the hyperplastic, virus-filled epidermal lesions, even in immunocompetent individuals (Senkevich *et al.*, 1997). Attempts to grow MCV in tissue culture or animals have been unsuccessful, but limited replication in human foreskin grafted to immunodeficient mice has been reported (Senkevich *et al.*, 1997). Although the lack of an *in vitro* replication system precluded characterization of MCV for many years, the determination of the genome sequence of MCV (Senkevich *et al.*, 1997), has allowed the comparison of gene sequences with other poxvirus genomes. This methodology has allowed the identification and study of a number of novel MCV genes (Bertin *et al.*, 1997; Krathwohl *et al.*, 1997; Shisler *et al.*, 1998) in the absence of MCV infection providing new insights into the MCV-host relationship.

Entomopoxvirinae

Insects are the only known hosts of the Entomopoxvirinae, and the observed viral host range is restricted to one or a few related species (Afonso *et al.*, 1999). Entomopoxvirinae are subdivided into three genera based primarily on differences in viral host range and virion morphology. Genus A viruses infect coleopterans, genus B viruses infect lepidopterans and orthopterans, and genus C viruses infect dipterans (Afonso *et al.*, 1999).

Melanoplus sanguinipes EPV (MsEPV), a Genus B virus infects the North American migratory grasshopper *M. sanguinipes*, an agriculturally important insect pest, in addition to two related grasshopper species (*M. differentialis* and *M. packardii*), the desert locust (*Schistocerca gregaria*), and the African migratory locust (*Locusta migratoria*) (Afonso *et al.*, 1999). MsEPV produces a large ellipsoid virion (250 to 300 nm in length) with a rectangular core. Grasshopper nymphs are infected by MsEPV after ingestion of virus-containing occlusion bodies (Afonso *et al.*, 1999) with the virus infecting cells of the midgut prior to generalization of infection to the major target organ, the fat body. Infection results in a slow and debilitating disease with high mortality occurring 25 to 30 days post-infection. High titers of infectious spheroids, which can number up to 8×10^7 per grasshopper, are evident at 12 to 15 days post-infection (Afonso *et al.*, 1999). EPVs have been studied mainly because they are potential insect biocontrol agents and expression vectors. Although the genome sequence of MsEPV has been recently determined, molecular mechanisms of EPV replication, pathogenesis, and host range are largely unknown (Afonso *et al.*, 1999).

Poxvirus Life Cycle

The study of poxviruses has been motivated by a desire to understand both the pathogenesis and the unique life cycle of these large complex DNA viruses (Moss, 1996a; Moss, 1996b). Detailed information regarding poxviruses has been derived mainly from studies with vaccinia virus, although the basic features may largely apply to other family members as well (Moss, 1996a; Moss, 1996b; Wittek, 1994). Infectious vaccinia virus particles are brick-shaped, measuring approximately 350 x 250 x 250 nm with lipoprotein membranes that surround a complex core structure containing a linear double stranded (ds) DNA molecule (Moss, 1996a). The two strands of vaccinia virus DNA are connected by

hairpin loops which form a covalently continuous polynucleotide chain (Moss, 1996b). The loops, which are A+T rich, cannot form a completely base paired structure and contain extra-helical bases (Moss, 1996b). The genome is further characterized by the presence of inverted terminal repeats (ITRs), which are identical but oppositely oriented sequences at the two ends of the genome (Moss, 1996b).

The vaccinia virus genome encodes approximately 200 proteins, many of which have not been assigned a precise function (Goebel *et al.*, 1990). The majority of polypeptides with known or suspected functions are enzymes involved in nucleic acid metabolism or transcription, which is consistent with the autonomy of these viruses and their cytoplasmic site of replication (Moss, 1996b). Examples include a multisubunit DNA-dependent RNA polymerase, capping and methylating enzymes, poly (A) polymerase, DNA polymerase, thymidine and thymidylate kinases, and a DNA ligase (Moss, 1996b). The total number of virion proteins representing both structural proteins and viral encoded enzymes which are packaged within the virus core may be as high as 100 (Wittek, 1994). In addition, viral encoded proteins may be subject to a variety of post-translational modifications such as glycosylation, phosphorylation, acylation and myristylation; for example, many envelope proteins are glycosylated, the 37 kDa major envelope protein is acylated and the membrane associated L1R polypeptide is myristylated (Moss, 1996b; Wittek, 1994).

Poxviruses are unique among DNA viruses in that their replication cycle occurs exclusively within the cytoplasm of the infected cell (Moss, 1996b). The first step after virus adsorption to the cell membrane is entry via fusion of the viral envelope with the host cell membrane (Moss, 1996b). A virally encoded protein with strong structural similarity to epidermal growth factor (EGF) has been found in vaccinia, and called VGF for vaccinia growth factor (Brown *et al.*, 1985). For vaccinia, the epidermal growth factor (EGF) receptor may function as the cellular receptor (Eppstein *et al.*, 1985). This, however,

remains controversial as conflicting evidence has been reported (Hugin and Hauser, 1994). Recent evidence suggests poxviruses such as myxoma virus may utilize cellular chemokine receptors for entry in a currently undefined mechanism, that is at least distinct from HIV entry (Lalani *et al.*, 1999). Following entry, the outer virion protein layers are lost and viral cores are released into the cytoplasm (Wittek, 1994).

VV Replication

The early viral encoded transcription system is packaged within the core of the infectious poxvirus particle. Following entry into the cytoplasm, virus cores synthesize early mRNA and then undergo a second uncoating step releasing the parental viral nucleoprotein complex (Moss, 1996b). DNA synthesis occurs and results in the generation of approximately 10,000 genome copies per cell of which half are ultimately packaged into virions (Moss, 1996b). Release and synthesis of the viral DNA allows expression of both intermediate and late genes, the transcription of which requires a naked DNA template (Sanz and Moss, 1999). Viral DNA replication occurs in precise regions in the cytoplasm termed virosomes or virus factories, that correspond to dense regions visible by electron microscopy or by optical microscopy after fluorescent labeling of DNA or protein components (Beaud, 1995).

Virally encoded enzymes with a known or presumed function in DNA replication include the E9L gene product, a 116 kDa DNA polymerase with intrinsic 5'-3' polymerization and 3'-5' exonuclease activity, a processivity factor, a DNA ligase (A50R), a thymidine kinase (J2R), a thymidylate kinase (A48R), a DNA topoisomerase I (H6R), small and large subunits of ribonucleotide reductase (F4L and I4L respectively) and a single stranded DNA binding protein (I3L) (Rochester and Traktman, 1998). Phenotypic analysis of temperature sensitive DNA- mutants has also revealed essential roles for the viral

encoded B1R serine/threonine protein kinase, D5R nucleoside triphosphatase and a uracil DNA glycosylase (D4R) in supporting viral DNA replication (Evans *et al.*, 1995).

Although large information gaps exist in the understanding of poxvirus DNA replication, the unique terminal structure of the poxvirus genome, the presence of concatemer junctions in replicating DNA, and the absence of a defined replication origin suggest a self-priming replication model (Moss, 1996b). The current model of poxvirus DNA replication involves the formation of concatamers; the formation of a hypothetical nick at one or both ends of the genome is followed by elongation of the DNA chain by viral DNA polymerase starting from the nick exposed 3'OH primer terminus. The inverted repeat thus formed, can fold back and continued DNA leading strand synthesis results in the formation of concatameric intermediates (Beaud, 1995; Traktman, 1990a). These concatamers are then resolved into unit length DNA molecules and are incorporated into virus particles at the late stage of infection (Beaud, 1995). Concatameric resolution is a highly specific process and depends on a 20 bp element located adjacent the hairpin loop in the mature DNA molecule (Wittek, 1994). DNA replication by itself does not seem to require specific origins of replication since any DNA transfected into vaccinia virus infected cells undergoes replication (Wittek, 1994).

VV Transcription

Vaccinia virus transcription is characterized by three temporal gene classes (early, intermediate and late) that are regulated by the presence of specific transcription factors made by the preceding temporal class of genes (Moss, 1996b). For example, early gene transcription factors are made late in infection and incorporated into virions for use in the subsequent round of infection. The promoters of early, intermediate and late stage genes are of similar length, however, each contain distinctive sequence elements that are recognized by

the specific viral transcription factor to provide the basis for a programmed, cascade mechanism of gene regulation (Baldick *et al.*, 1992; Davison and Moss, 1989a; Davison and Moss, 1989b). The RNA polymerase of vaccinia virus contains 8 virus-encoded subunits (Amegadzie *et al.*, 1992; Baroudy and Moss, 1980). The two largest and the smallest subunits are homologous to the corresponding size subunits of eukaryotes and another is homologous to a eukaryotic transcriptional elongation factor (Amegadzie *et al.*, 1992). Specific promoter recognition is governed by the interaction of stage-specific viral encoded transcription factors with the multisubunit viral RNA polymerase (Sanz and Moss, 1999).

Initially, only the early genes are transcribed: they encode proteins involved in stimulation of the growth of neighboring cells (VGF), defense against host immune responses, replication of the viral genome, and transcription of the intermediate class of viral genes. The vaccinia virus early transcription factor (VETF), which possesses DNA-dependent ATPase activity (Broyles and Moss, 1988), and a 94 kDa (Rap 94) protein which confers early promoter specificity (Ahn *et al.*, 1994), are synthesized at late times after infection and packaged along with the multisubunit RNA polymerase, such that transcription of early genes occurs immediately after infection and does not require *de novo* protein or DNA synthesis (Moss, 1996b).

The early stage mRNAs are of a discrete size and are capped, methylated and polyadenylated similar to eukaryotic mRNAs (Wittek, 1994). The cap structure is formed on the nascent RNA by two virus-encoded enzymes: the first, commonly called capping enzyme, is a heterodimeric protein with RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7-)-methyltransferase activities; the second is an RNA (nucleoside-2'-)-methyltransferase which exists both as a 39 kDa protein and as a subunit of the poly(A) polymerase(Moss, 1996b). The capping enzyme also has a role in transcription termination

of early mRNAs, which occurs about 20 to 50 nucleotides after the sequence UUUUUNU (where N is any nucleotide) in mRNA (Moss, 1996b). This sequence does not signal termination of intermediate or late transcripts, which are heterogeneous in length. Polyadenylylation is performed by a virus-encoded heterodimeric protein: one subunit (VP55) has adenylyltransferase activity and the other (VP39) stimulates elongation (Moss, 1996b).

The viral proteins that mediate transcription of intermediate stage genes are synthesized before DNA replication (Moss, 1996b). Intermediate promoters are characterized by the sequence TAAA at the initiator site (Baldick *et al.*, 1992). Late gene promoters contain the highly conserved TAAATG/A motif in which transcription initiation occurs (Davison and Moss, 1989b; Moss, 1996b). Late mRNAs are heterogeneous in length due to transcriptional readthrough, are polyadenylated and have a capped poly(A) leader sequence of about 35 A residues (Moss, 1996b; Wittek, 1994). Regulation of vaccinia virus gene expression occurs primarily at the transcriptional level (Moss, 1996b). Viral mRNAs are translated on the cytoplasmic polysomes (Wittek, 1994).

Assembly and Dissemination

Upon synthesis of the late structural proteins, infectious virus particles are assembled and acquire an envelope (Wittek, 1994). This complex process, which requires several hours for completion is poorly understood (Wittek, 1994). Some of these particles migrate along actin-containing microfilaments to the cell surface where they bud through the plasma membrane and either remain attached to the cell surface or are released into the medium (Moss, 1996a). The externalized forms of vaccinia virus are generally thought to mediate cell to cell spread (Moss, 1996a).

VV infection results in rapid shut-off of host cell DNA, RNA and protein synthesis and the virus exhibits a large degree of autonomy from host cellular functions (Moss, 1996b). However, there is some active cellular contribution to the viral life cycle because in enucleated cells, while poxvirus gene expression and genome replication occurs, the process of viral maturation is blocked (Villarreal *et al.*, 1984).

Poxviral Immune Evasion

Restriction fragment length polymorphism (RFLP) analysis and comparison of published poxviral genome sequences has revealed that in general viral encoded genes necessary for transcription, replication and assembly of the virus particles are well conserved among different poxvirus families and cluster in the central region of the genome (Robinson and Mercer, 1995; Traktman, 1990b). In contrast, the terminal regions of the genome show marked variability in sequence between families and even among the same genus (Traktman, 1990b). In many cases, these genes can be disrupted without affecting the replicative ability of the virus in tissue culture, however, frequently they are found to determine viral host range, tissue specificity, replication and virulence within the natural host (Upton *et al.*, 1992; Upton *et al.*, 1994).

The examination of such genes and their gene products can provide valuable information not only about the viral-host interactions occurring during infection but also about the antiviral response of the host's immune system in general (Upton *et al.*, 1994). The large poxviral genome has facilitated the encoding of a large repertoire of viral defense molecules to circumvent host immune cell function (Wall *et al.*, 1998). These virulence factors frequently increase the manifestation of disease symptoms in animal models (Wall

et al., 1998). Many of the host defense molecules are related in sequence to eukaryotic proteins, suggesting that they were acquired from the host during evolution (Wall *et al.*, 1998).

Interaction of an infectious agent with host immune surveillance cells, such as macrophages or antigen specific lymphocytes, elicits an immune response that is largely orchestrated by a broad family of soluble cellular signaling peptides termed cytokines (Biron, 1994). Poxviruses encode numerous secreted proteins which block cytokine mediated communication within the immune system (Upton *et al.*, 1991). These viral proteins, termed viroceptors, often possess significant similarity to the ligand binding domain of the cellular cytokine receptor and function as receptor mimics (Wall *et al.*, 1998). By binding their respective ligands, these viroceptors disrupt the normal intracellular signaling pathways induced by cognate receptor binding. Examples include viral proteins that target and counteract immune molecules of the host such as TNF (Upton *et al.*, 1991), interferon-γ (Upton *et al.*, 1992), interleukins (Spriggs *et al.*, 1992) and chemokines (Cao *et al.*, 1995; Graham *et al.*, 1997). Additionally, poxviruses have evolved supplementary defense mechanisms that act intracellularly on such signaling pathways, providing a multi-faceted approach to cytokine disruption.

(1) Tumor necrosis factor (TNF)

TNFα and TNFβ are closely related cytokines, produced by macrophages and lymphocytes respectively, which exhibit potent anti-viral activity and play pivotal roles in the regulation of immune system function (Ware *et al.*, 1996). TNFα and TNFβ bind

membrane associated cellular receptors TNFR-I (TNFR60) and TNFR-II (TNFR80) respectively (Ware *et al.*, 1996). The T2 protein of SFV is secreted as a soluble glycoprotein that specifically binds both radiolabeled TNFα and TNFβ (Smith *et al.*, 1991). Deletion of the T2 gene from the closely related MYX resulted in significant attenuation of this mutant virus in rabbits, suggesting TNF plays a major role in combating MYX infection *in vivo* (Upton *et al.*, 1991).

The mechanism of action of the SFV T2 protein may not be restricted to dysregulation of the cellular immune TNF response, but may also stem from modulation of cellular apoptosis in response to infection (Sedger and McFadden, 1996). Expression of MYX T2 protein has also been demonstrated to reduce apoptosis or programmed cell death of infected rabbit lymphocytes in a mechanism which is independent of TNFα binding (Schreiber *et al.*, 1997). This suggests elements of the T2 protein that are distinct from the cysteine rich ligand binding domain may function to negatively regulate intracellular apoptotic molecules downstream of TNF receptor signaling.

(2) Interferons

In addition to their antiviral effects, interferons also function as potent immunomodulatory molecules. IFNs, represent a large family of proteins that are broadly categorized into two groups: Type I IFNs, α and β, are induced by virus infection of most somatic cells, whereas type II IFN (IFN-γ) is produced by T lymphocytes and natural killer (NK) cells following mitogenic and antigenic stimulation (Sen and Lengyel, 1992). IFN-γ acts as a key regulatory cytokine molecule that promotes the differentiation of myeloid cells, macrophage activation and the expression of major histocompatability (MHC) molecules on the surface of a variety of cells (Billiau *et al.*, 1998). Type I interferons bind to specific receptors on neighboring uninfected cells and induce transmembrane signaling events that

ultimately lead to the transcription of genes involved in promoting an antiviral state (Kaufman, 1999). This antiviral state primes the uninfected cell such that following viral infection, viral replication and assembly are prevented.

Antiviral interferon action is mediated by more than one process. In response to the presence of dsRNA within the cell, activation of protein kinase p68 (PKR) catalyses the phosphorylation of eukaryotic translation initiation factor 2 on Ser-51 of its α subunit (eIF2α). Protein synthesis is blocked and activation of 2-5-linked oligoadenylate (2-5A) synthetase leads to the formation of a short polymer of adenylic acid that serves to activate a latent cellular ribonuclease (RNaseL) that nonspecifically degrades single stranded RNA (Kaufman, 1999). Poxviruses have evolved several mechanisms to counteract interferon action. The VV B18R gene encodes a type I interferon receptor which is secreted from infected cells and binds to the surface of both infected and uninfected cells (Colamonici *et al.*, 1995) by an undetermined mechanism. B18R specifically binds IFN-α from several species (Symons *et al.*, 1995) and thereby prevents IFN-α receptor stimulation such that IFN-α inhibition of vesicular stomatitis virus (VSV) replication is circumvented (Colamonici *et al.*, 1995). Complete orthologs of this gene are found in a number of poxviruses and disruption of the B18R gene within VV results in attenuation of virulence in mice (Symons *et al.*, 1995). The MYX M-T7 gene encodes a viral IFN-γ receptor protein that is secreted in large amounts from MYX infected cells (Upton *et al.*, 1992). M-T7 protein specifically binds rabbit IFN-γ and blocks its ability to protect cells from VSV infection (Upton *et al.*, 1992). A number of orthologs have been identified in a variety of other poxviruses. The importance of IFN-γ in resolution of poxviral infections is evidenced by attenuation of MYX virulence following deletion of the M-T7 gene (Mossman *et al.*, 1996). Within the infected cell, poxviruses also need to counteract the IFN response. The terminal heterogeneity of poxviral late mRNA transcripts, combined with transcription from both DNA strands results in some mRNAs annealing to produce dsRNA (Moss, 1996b).

The E3L gene product of VV-WR, a dsRNA binding protein, effectively binds and sequesters dsRNA such that both activation of PKR and 2-5-linked oligoadenylate (2-5A) synthetase by dsRNA is inhibited (Chang *et al.*, 1992; Rivas *et al.*, 1998). Additionally, VV encodes an eIF-2α homologue, the product of the K3L gene, which may interfere with the cessation of protein synthesis promoted by activated protein kinase p68 by competing with the cellular eIF-2α for kinase binding and phosphorylation (Davies *et al.*, 1993). K3L reduces the level of phosphorylated eIF-2α in VV-infected cells and disruption of the K3L gene from VV, sensitizes the virus to the effects of IFN treatment (Beattie *et al.*, 1995).

(3) Interleukins

Interleukins are a family of pleiotrophic cytokines that function in a variety of processes including inflammation, chemoattraction, immune cell activation and proliferation (Kelso, 1998). Interleukin 1 (IL-1) is a primary regulator of inflammatory and immune responses (Stylianou and Saklatvala, 1998). Via its type I receptor, it activates specific protein kinases, including the NFκB inducing kinase (NIK) and three distinct mitogen-activated protein (MAP) kinase cascades (Stylianou and Saklatvala, 1998). These modulate a number of transcription factors including NFκB, AP1 and CREB, each of which regulates a plethora of immediate early genes central to the inflammatory response (Stylianou and Saklatvala, 1998).

The VV B15R gene product possesses significant similarity to the external ligand binding domain of cellular interleukin-1 receptors. VV B15R is secreted from infected cells as a 50-60 kDa glycosylated protein and binds functional mature IL-1β (Alcami and Smith, 1992; Spriggs *et al.*, 1992). Additionally, the MCV genome is found to encode three genes (MC51L, MC53L, and MC54L) that encode glycosylated secreted homologues of the recently discovered human IL-18 binding proteins (Xiang and Moss, 1999). Human and

mouse secreted IL-18 binding proteins are distinct from membrane IL-18 receptors and antagonize IL-18 activity (Smith *et al.*, 2000). IL-18 is a proinflammatory cytokine that induces synthesis of IFN-γ, activates NK cells, and is required for a T-lymphocyte helper type 1 response (Xiang and Moss, 1999). Recombinant MC53L and MC54L proteins bind both human and murine IL-18 with high affinity and inhibit IL-18 mediated IFN-γ production in a dose dependent manner (Xiang and Moss, 1999). This suggests that these viral proteins antagonize the development of an inflammatory response to MCV infection in humans. Viral secreted IL-18 binding proteins are also conserved in EV, VV and CPV (Smith *et al.*, 2000). Interestingly, recombinant baculovirus expressed EV IL-18 binding protein is found to block NF-κB activation and induction of IFN-γ in response to IL-18 (Smith *et al.*, 2000), consistent with a proposed role for this viral modulator in suppressing inflammation.

At the intracellular level, poxviruses are found to interfere with the processing and functional maturation of IL-1. Cleavage of pro-IL-1β by a cysteine protease, designated IL-1β converting enzyme (ICE or caspase-1), produces the mature secreted 17.5 kDa form of IL-1β. CPV and other poxviruses encode a serine protease inhibitor (serpin) homologue designated crmA which complexes caspase-1, and thereby blocks the maturation of pro-IL-1β and the host inflammatory response to infection (Ray *et al.*, 1992). Disruption of the CPV crmA gene results in the formation white pocks instead of the wildtype red hemorrhagic pocks on the chorioallantoic membranes of chicken embryos (Pickup *et al.*, 1986). The white pock phenotype results from a failure to prevent an influx of inflammatory cells (mainly heterophils and macrophages) into the developing lesion (Fredrickson *et al.*, 1992; Palumbo *et al.*, 1989). Additionally, CPV crmA expression has a major role in protecting the infected cell from apoptosis promoted by a variety of agents such as TNF, anti-Fas antibody, cytotoxic-T-lymphocyte activation and granzyme B (Dou *et al.*, 1997; Quan *et al.*, 1995; Tewari and Dixit, 1995; Tewari *et al.*, 1995c).

(4) Chemokines

Chemokines (chemotactic cytokines) are small 70 to 80 amino acid proteins with a characteristic dicysteine motif that are produced by lymphocytes and a variety of other cells (Krathwohl *et al.*, 1997). They are involved in attracting and activating distinct leukocyte subsets to inflammatory foci and specific tissues and microenvironments within tissues such as in a lymph node (Luttichau *et al.*, 2000). The precise number of human chemokines is unclear, but is currently thought to be upwards of 50 (Luttichau *et al.*, 2000). Depending on the presence and spacing of the two N-terminal cysteine residues, they are classified into subfamilies CXC, CC, C and CX3C (Carfi *et al.*, 1999). In general, CXC chemokines attract neutrophils or lymphocytes and CC-chemokines attract monocytes, lymphocytes, eosinophils or basophils, whereas lymphotactin (the only member of the C-chemokines) attracts T and natural killer (NK) cells (Carfi *et al.*, 1999). Chemokines exert their function through seven-transmembrane (7TM) G-protein-coupled receptors and individual members tend to exhibit specificity for members of the same chemokine subfamily (Carfi *et al.*, 1999).

Poxviruses encode a family of 35 kDa secreted soluble proteins termed vCCI (viral CC-chemokine inhibitor) or vCKBP (viral chemokine binding proteins) that bind, with subnanomolar dissociation constants, to CC-chemokines, but not to CXC- or C-chemokines (Alcami *et al.*, 1998; Graham *et al.*, 1997; Smith *et al.*, 1997). vCCIs or vCKPBs are encoded by EV (Wall *et al.*, 1998), CPV, rabbitpox, raccoonpox, camelpox, VAR, VV (strain Lister), SFV and MYX viruses and remarkably exhibit no sequence homology with known host chemokine receptors or any other known proteins (Alcami *et al.*, 1998; Graham *et al.*, 1997; Smith *et al.*, 1997). Deletion of the gene encoding this chemokine binding protein (T1/35kDa) from rabbitpox virus resulted in an increased number of extravasating

leukocytes in the deep dermis following infection of rabbits (Graham *et al.*, 1997), indicating that the secreted poxviral chemokine binding proteins likely function by competing with cellular chemokine receptors for chemokine binding, and thereby retard the activation and chemotaxis of monocytes in the early stages of the host inflammatory response to viral infection (Carfi *et al.*, 1999).

Additionally, MCV encodes a secreted CC-chemokine homologue (MC148) that binds to human chemokine receptors, and interferes with the chemotaxis of human monocytes, lymphocytes and neutrophils triggered by a large number of CC and CXC chemokines with diverse receptor specificities (Damon *et al.*, 1998). This viral secreted chemokine homologue may function as a receptor antagonist because the amino terminal region, which normally activates the receptor, is truncated in the MC148 gene product (Krathwohl *et al.*, 1997). In support of this hypothesis, MC148 receptor binding does not result in intracellular Ca2+ influx, an essential step in chemokine activation (Damon *et al.*, 1998). Thus, MC148 can bind to chemokine receptors and block binding of host chemokines, but does not trigger the receptor. Recent evidence suggests MC148 selectively binds the cellular chemokine receptor CCR8 and may interfere with monocyte invasion and dendritic cell function at the site of infection (Luttichau *et al.*, 2000). These collective strategies may explain the characteristic absence of surrounding inflammatory cell infiltrates in MCV lesions.

(5) Complement system

In response to viral infection, the inevitable activation of complement, either by the classical pathway or principally by the alternative pathway, forms a major immune defense

mechanism. In addition to promoting a membrane attack complex (MAC), potent chemotactic complement factors such as C3a, C4a and C5a are released (Kotwal, 2000). One of the major VV encoded secreted proteins is a complement control protein termed VCP (Kotwal *et al.*, 1990) that contributes to virus virulence through the prevention of antibody-dependent complement-enhanced viral neutralization (Isaacs *et al.*, 1992). Vaccinia virus VCP is homologous to complement receptor 1 (CR1) and other mammalian regulators of complement activation (Kirkitadze *et al.*, 1999; Kotwal *et al.*, 1990). VCP binds to complement components C3b and C4b resulting in blockage of the formation of the C3 convertase complex, a crucial initial step following complement activation (McKenzie *et al.*, 1992). This inhibition not only diminishes the formation of the potent chemotactic factor C5a, but also circumvents the potential formation of the membrane attack complex (MAC) on the viral surface or the membrane of infected cells (Kotwal, 2000). The CPV homologue of the VV complement control protein is termed the inflammation modulatory protein (IMP) (Miller *et al.*, 1997). Infection of mice with a recombinant CPV lacking the homologue of VCP results in greater tissue damage, with more hemorrhage and induration and a greater prolonged specific swelling response compared to wild-type CPV infection (Miller *et al.*, 1997). This suggests that viral evasion of the complement system promotes down-regulation of the viral induced inflammatory response.

(6) Other Mechanisms

Additionally, there are a number of poxvirus immune modulating activities that have not yet been associated with a specific gene product. For example, MYX triggers CD4 down-regulation following infection of CD4+ T lymphocytes (Barry *et al.*, 1995). This MYX CD4 down-regulation is proposed to occur via a protein kinase C-independent pathway that results in the dissociation of p56lck from CD4 and the degradation of CD4 in lysosomal vesicles (Barry *et al.*, 1995). The HIV-1 encoded vpu, an integral membrane

protein largely responsible for the decrease in the expression of major histocompatibility complex (MHC) class I molecules on the surface of HIV-1-infected cells (Kerkau *et al.*, 1997), promotes degradation of CD4 in the ER of infected cells (Vincent *et al.*, 1993). It will be interesting to determine whether the MYX M-T4 gene product, a novel RDEL-containing protein that is retained within the endoplasmic reticulum (ER) (Barry *et al.*, 1997) similarly promotes degradation of CD4 in the ER.

Significance of Poxvirus Research

The study of poxviruses has been and continues to be a highly worthwhile endeavor. Poxviruses were the first viruses to be seen with a microscope, and Edward Jenner's work pioneered vaccination as we know it today. The study of poxviral virulence factors continues to have major ramifications for insight into diverse fields of study. For example, the identification of the poxviral crmA protein as an inhibitor of ICE promoted a search for other cellular ICE-like proteins and helped identify the family of cysteine proteases termed caspases, which are now known to be responsible for the critical proteolytic cleavage events that occur during apoptosis.

Biotechnological and medical applications resulting from of the study of poxviral virulence factors are apparent. For example, the observation of a poxvirus encoded receptor that sequesters cellular TNF, and thus reduces its proinflammatory effects (Smith *et al.*, 1990; Smith *et al.*, 1991), has been the intellectual basis of a novel therapeutic intervention strategy for rheumatoid arthritis (Garrison and McDonnell, 1999). Rheumatoid arthritis is an autoimmune disease characterized by excessive TNF production at inflammation sites such that the naturally occurring TNF receptors in the patient cannot adequately regulate TNF activity (Jeong and Jue, 1997). Developed by Immunex Corporation, Seattle, WA under the tradename ENBREL® (etanercept), a recombinant soluble TNF receptor is

administered intravenously and helps alleviate characteristic symptoms such as tender and swollen joints (Moreland *et al.*, 1999; Trehu *et al.*, 1996). This technology has been so successful, that recently it has been additionally approved by the US Federal Drug Administration (FDA) for the treatment of juvenile arthritis (Garrison and McDonnell, 1999; Lovell *et al.*, 2000).

Presently, a number of poxviral proteins have documented roles in virulence. However, they have no identified host homologues and as such clues to their specific roles remain largely speculative. The analysis of these poxviral proteins may shed new light on poxviral-host interactions or identify new cellular proteins active in the immune response to infection.

Dissertation Outline

The focus of this Ph.D. dissertation is a poxviral virulence factor. The poxviral RING finger protein family represents a family of proteins that exhibits no appreciable sequence similarity to known proteins except for the presence of a cysteine rich, zinc binding motif termed a RING finger at their C-terminus. They are found to localize to the virus factories within the cytoplasm of the infected cell. In beginning this research effort, a number of questions were evident, such as how these proteins were targeted to the factories, the precise function of the RING finger motif and how expression of the EV p28 gene was critical for viral pathogenesis. Studies presented here, have identified a DNA binding activity for the SFV RING finger protein and mutagenesis studies have identified sequence requirements of N1R in involved in both DNA binding activity and thus localization of N1R to the virus factories. These studies are presented in Chapter 1 of this dissertation.

Additionally, evidence is presented that N1R and the EV ortholog, p28, plays a role in the modulation of apoptosis following infection. These studies are presented in Chapter 2 of this dissertation. In order to provide clarity and structure, each part of this dissertation is introduced and discussed separately initially. The dissertation will close with a concluding overall discussion of results presented in both Chapters 1 and 2 of the dissertation and provide possible direction for future studies.

Contributors to Work Presented in this Thesis.

I am indebted to the following individuals for their contribution to work presented in this thesis

a) Dr. Robert D. Burke (Department of Biology, University of Victoria, Victoria, British Columbia, Canada) performed 1) confocal and immunofluorescence microscopic analysis on the localization pattern of SFV N1R and constructed N1R mutant proteins within VV infected cells 2) DAPI fluorescence analysis for the detection of apoptotic nuclei and DAPI staining of virus factories within VV infected cells.

b) Dr. Leslie Schiff (Department of Microbiology, University of Minnesota, Minneapolis, Minnesota, USA) performed *in vitro* zinc binding assays.

c) Dr. Chris Upton (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) performed alignments and structural prediction analysis.

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f) Mr. Aaron A. Minkley (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) performed experiments on the effects of UV light exposure on the number of virus progeny isolate from wild type and mutant EV infected HeLa cells, and aided in virus infections, tissue culture propagation, transfections and isolation of recombinant VV.

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i) Ms. Yasanna Quin (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) aided in tissue culture propagation and transfections.

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CHAPTER 1: Identification of Regions of the Shope Fibroma Virus RING Finger Protein N1R Required for Virus Factory Localization and DNA Binding Activity.

INTRODUCTION

Poxvirus RING finger proteins are characterized by the presence of a C-terminal zinc binding RING finger motif and by localization to the sites of poxvirus replication, known as virus factories or virosomes, within the cytoplasm of infected cells. Although non-essential for replication in a variety of tissue culture cells lines, poxviral RING finger proteins are associated with virus replication and virulence *in vivo*. This family of poxviral proteins appear to be unrelated to host proteins except for the cysteine rich zinc binding motif known as a RING finger. This motif has enjoyed considerable attention because of its association with human disease; however, its precise function has remained controversial. In contrast to zinc finger motifs in transcription factor DNA binding proteins, the RING finger motif appears to be involved in mediating protein-protein interactions and recently this motif has been implicated in the process of ubiquitination. Studies presented here indicate a role for the RING finger motif in localizing the protein to the viral factories; however, regions of the poxviral RING finger proteins which are distinct from the RING finger motif and which may form a ribbon-helix-helix DNA interaction motif are required for association of the proteins with DNA cellulose and the cytoplasmic viral factories.

SFV and MYX RING finger proteins

In 1994, the sequencing of the *Bam*HI N fragment of the SFV DNA genome identified the N1R open reading frame (ORF). The gene was predicted to encode a novel 28

kDa protein. Except for the presence of a RING zinc finger motif at its C-terminus, the N1R protein had little sequence similarity with non-poxviral proteins (Upton *et al.*, 1994). Complete orthologs of this gene are present in a number of other poxviruses including MYX (m143R) (Cameron *et al.*, 1999; Upton *et al.*, 1994), EV (p28) (Senkevich *et al.*, 1994), VAR (D4R) (Shchelkunov *et al.*, 1994), CPV (D7R) (Safronov *et al.*, 1996), FPV (FPV150) (Afonso *et al.*, 2000) and VV strain IHDW (Upton *et al.*, 1994). The gene, however, is absent from the genome of VV strain Copenhagen (Goebel *et al.*, 1990), and in VV strain WR the ORF (designated 21.7K *Hind*III-C) is truncated because of an 11 bp deletion which produces a frameshift and a premature stop codon following Cys2 of the RING zinc finger motif (Kotwal and Moss, 1988).

The SFV N1R protein was predicted to bind zinc ions because of the RING motif. This was confirmed experimentally for the SFV N1R protein and the MYX ortholog, expressed in *E. coli* using a zinc blot assay (Upton *et al.*, 1994). Localization studies were performed using a monoclonal antibody (Mab H1119) directed to an epitope tag fused to the N-terminus of the poxvirus proteins with detection by fluorescence microscopy. It was found that the N1R proteins of SFV, MYX and VV-IHDW localized to discrete regions within the cytoplasm of poxvirus infected cells corresponding to the viral factories or virosomes (Upton *et al.*, 1994) which are the sites of poxviral DNA replication (Traktman, 1990a). Although the truncated N1R protein from VV-WR was stable, it did not localize to the factories but was observed distributed throughout the cytoplasm of infected cells (Upton *et al.*, 1994).

EV RING finger protein (p28)

The EV ortholog (p28) of SFV N1R has also been described and characterized as a zinc binding virus factory-associated protein (Senkevich *et al.*, 1994; Senkevich *et al.*, 1995). By

expressing 18 kDa N-terminal and 10 kDa peptide C-terminal fragments of the p28 protein in *E. coli* and using an *in vitro* zinc binding assay, zinc binding was attributed to the C-terminal 10 kDa peptide containing the RING finger motif (Senkevich *et al.*, 1994).

A p28- knockout mutant EV was constructed using a vector in which the C-terminal region of p28 encoding the RING finger motif, was replaced with the xanthine-guanine phosphoribosyltransferase (gpt) selectable marker, leaving the 5' terminus of the p28 gene (encoding the first 152 amino acids) intact. Experiments with this mutant EV demonstrated that p28 expression was non-essential for the multiplication of EV in several tissue culture cell lines including BSC-1 (African green monkey cells), primary mouse ovary cells, primary mouse embryo fibroblasts and Raw 264.7 cells (mouse monocyte/macrophage cells) (Senkevich *et al.*, 1994). The ability to generate the p28-mutant EV in BSC-1 cells as well as the failure to find a requirement for p28 in the replication cycle of EV in several tissue culture cells, suggested that p28 functioned during virus infection of the natural host, the mouse. Infection of specific pathogen free female ANCR (A) mice with this p28-mutant EV revealed that expression of wild-type p28 was critical for EV pathogenicity in these A strain mice (Senkevich *et al.*, 1994). Mice infected through the footpad with wild type EV (5 pfu) showed a uniform mortality with a mean day of death of 9.7 days post infection. The EV dose required to cause a lethal infection in 50% of test animals (LD_{50}) was calculated to be 0.25 PFU. In contrast, all mice infected with the p28- mutant EV survived the challenges, with little or no morbidity. The LD_{50} was calculated to be greater that 2.5×10^5 PFU (Senkevich *et al.*, 1994).

The p28- mutant EV virus replicated to significantly lower titers than the wild type virus in different organs of infected mice (Senkevich *et al.*, 1994). Interestingly, the amount of p28-mutant EV that could be isolated from the footpad injection site was less than the amount of EV-WT recovered from infected mice as early as 1 day post-infection. At 14

days post-infection no p28-mutant EV was detectable, suggesting that host defense mechanisms had successfully cleared the mutant virus (Senkevich *et al.*, 1994). The dramatic difference in the LD_{50} of EV and mutant p28- EV was correlated with the difference in the number of progeny virus isolated from livers and spleens following footpad inoculation (Senkevich *et al.*, 1994).

Virus pathogenesis is shaped by complex interactions between virus encoded virulence factors, virus replicative ability and specific and non-specific host immune responses. Similar to the replication of the EV and the p28- mutant virus in the A strain of mice, infection of athymic nude mice (characterized by a severely depressed number of T-cells) and severe combined immunodeficiency (SCID) mice (characterized by a depressed numbers of B- and T- cells) revealed a requirement for p28 expression in promoting EV replication from the site of inoculation to the target organs, the liver and spleen (Senkevich *et al.*, 1995). Surprisingly, the difference in replication of the p28- mutant virus compared to EV was more pronounced in the footpad, spleen and liver of athymic mice as compared to SCID mice. Infection of SCID mice with wild type EV resulted in death at 9 days post-infection. It is noteworthy, that infection of SCID mice with the p28- mutant EV also resulted in death of these mice, however, the day of death occurred 5 days later than the wildtype EV infection (Senkevich *et al.*, 1995). This suggests a potential role for p28 in promoting EV virulence through enhancement of both virus replication and immune evasion.

In agreement with the localization studies carried out on SFV N1R, immunofluorescence and biochemical analyses found that p28 associated with the virus factories in EV-infected macrophages or BSC-1 cells. After examination of a small set of C-terminal truncated mutants of p28, it was reported that disruption of the RING finger motif had no influence on the intracellular localization of p28 (Senkevich *et al.*, 1995). Studies using polyclonal antisera raised against *E. coli* expressed p28 further indicated that p28 is

an early viral protein, detectable by western blot analysis as soon as 2 hours post-infection, that continues to accumulate throughout the infection for at least 24 hours (Senkevich *et al.*, 1995). Furthermore, p28 is thought not to be incorporated into virus particles, since it was not detectable by western blot analysis of extracts of purified EV virions (Senkevich *et al.*, 1995).

A possible clue to the role of p28 in promoting EV virulence came from a report that indicated the expression of p28 was required for *in vitro* replication of EV in murine resident peritoneal macrophages (Senkevich *et al.*, 1995). In contrast to wild type EV infection, viral DNA replication was not detected in macrophages infected with the p28- mutant EV and following Hoechst dye staining, no factories were found in most of the p28- mutant infected cells. The synthesis of at least two viral early proteins, however, was observed following infection of macrophages with the p28- mutant EV (Senkevich *et al.*, 1995). It remains unclear from this report, however, whether p28 expression is required for formation of the virus factories in these infected macrophages. If so, then this observation is likely not a generality, as the p28- mutant EV, which produces a truncated non-factory localizing protein (Senkevich *et al.*, 1995) replicated to undistinguishable levels compared to EV in a variety of cell lines (Senkevich *et al.*, 1994; Senkevich *et al.*, 1995).

It has been hypothesized that in macrophages, which are highly specialized nondividing cells, p28 substitutes for an unknown cellular factor(s) that is required for EV DNA replication or a stage of virus reproduction between the expression of early genes and the onset of DNA synthesis. Further, it was proposed that the attenuation of the p28- mutant EV in mice was due to the failure of the virus to replicate in macrophage lineage cells at all successive steps in the spread of virus from the skin to its target organ, the liver (Senkevich *et al.*, 1995). This hypothesis, however, conflicts with the observation that the p28 mutant EV replicated and spread from the site of inoculation to the target organs in SCID mice. Overall, these studies utilizing the p28-mutant EV suggested that the C-terminal sequence of

p28, including the RING finger motif, has a role in both promoting poxviral DNA replication in macrophages and EV virulence in the natural host.

Zinc Finger Motifs

Zinc finger motifs are best known as transcription factor DNA binding domains. They are small autonomously folding and functional protein domains stabilized by zinc ions. The tandem repetition of these structurally identical but chemically distinct units specifies a modular system for the recognition of a specific DNA sequence (Schwabe and Klug, 1994). Zinc finger proteins are very widespread in nature (Mackay and Crossley, 1998) and the zinc finger domains of DNA binding proteins can be grouped into three classes.

The first class or prototypical "zinc finger" was discovered in the transcription factor IIIA (TFIIIA) of *Xenopus laevis* (Rhodes and Klug, 1993). Proteins in this class usually contain tandem repeats of a 30 amino acid zinc finger motif (Cys-X(2 or 4)-Cys-X(12)-His-X(3-5)-His; where X is any amino acid). Structural determination of TFIIIA indicated that these zinc fingers consist of an antiparallel β-sheet and a α-helix. Two cysteines, which are near the turn in the β-sheet region, and two histidines, which are in the α-helix, coordinate a central zinc ion and hold these secondary structures together to form a compact globular domain (Pabo and Sauer, 1992). The crystal structure of a zinc finger-DNA complex containing three fingers of the transcription factor zif268 and a consensus zif binding site showed that the zinc finger wraps partly around the DNA helix and utilizes the α helix of each finger to make contacts with successive 3 bp sites in the major groove of DNA (Pavletich and Pabo, 1991).

The second class of zinc binding domains are the steroid receptors. The approximately 70 amino acid DNA binding domains of these receptors have eight conserved cysteine residues that originally prompted the proposal that this region might form a pair of zinc fingers. The steroid receptors, however, were found to form a distinct structural motif. NMR analysis revealed that the receptors fold into a single globular domain with a pair of α-helices, roughly perpendicular to each other, held together by hydrophobic contacts. Each zinc ion is tethered by four cysteines residues, near the start of each α helix, and holds a peptide loop against the N-terminal end of the helix (Schwabe and Rhodes, 1991). The crystal structure of the glucocorticoid receptor complex demonstrated that the receptor binds DNA as a dimer, with the first helix of its DNA binding domain lying in the major groove, and that side chains from the second and third turns of this helix directly interact with the nucleotide bases (Luisi *et al.*, 1991). Thus, the consensus DNA elements that these receptors recognize have a two-fold symmetry.

The third major class of zinc binding domains is found in yeast transcriptional activators. The prototype, Gal4, binds as a dimer to a 17 bp site with twofold symmetry (Harrison, 1991). In Gal4, six cysteines interact with two zinc ions to form a binuclear zinc thiolate cluster. Thus, in this novel structure, each zinc ion is coordinated by four cysteines with two of the six cysteines being shared (Vallee *et al.*, 1991).

DNA binding motifs are diverse and many await characterization. They are not, however, restricted to the utilization of protruding surface helical regions for direct interaction with the nucleotide bases of DNA. For example, the ribbon-helix-helix family of proteins utilizes a short β sheet for specific DNA interaction (Lum and Schildbach, 1999; Suzuki, 1995). This class of proteins includes the Arc and Mnt repressor proteins of Salmonella phage P22 (Knight *et al.*, 1989b; Raumann *et al.*, 1994), the MetJ repressor of *E. coli* (Somers and Phillips, 1992; Somers *et al.*, 1994), the F Factor TraY gene product (Lum and Schildbach, 1999) and the recently described *Pseudomonas aeruginosa*

transcriptional activator AlgZ (Baynham *et al.*, 1999). Members of this family contain an N-terminal motif consisting of a β-strand followed by two α helical regions that are important for oligomerization and facilitating direct interaction of the β-sheet with DNA (Suzuki, 1995).

RING finger motif

It has become increasingly evident that zinc binding motifs are not, however, solely restricted to DNA binding activity and may also function in mediating protein-protein interactions (Mackay and Crossley, 1998). In 1993, the Really Interesting New Gene 1 or RING1 was identified proximal to the major histocompatibility complex (MHC) region on human chromosome 6 (Lovering *et al.*, 1993). Sequence analysis identified a novel cysteine-rich motif within the N-terminal region of RING1 that was conserved in a number of otherwise unrelated proteins (Freemont *et al.*, 1991). Presently, this motif is known as the RING zinc finger or C3HC4 motif and has been described in upwards of 200 proteins (Freemont, 2000). The RING finger family is evolutionarily diverse, comprising proteins from plants, viruses and humans (Saurin *et al.*, 1996).

RING fingers are cysteine-rich zinc-binding domains characterized by a pattern of conserved cysteine and histidine residues (Borden and Freemont, 1996; Saurin *et al.*, 1996). The RING finger motif has been defined simply as Cys-X(2)-Cys-X(9-39)-Cys-X(1-3)-His-X(2 to 3)-Cys-X(2)-Cys-X(4-48)-Cys-X(2)-Cys (PROSITE: PS00518, PDOC00449) where X is any amino acid. In addition to the highly conserved cysteines and histidine residue, there is also a preference for hydrophobic residues before Cys2 and Cys4 and after Cys5 and Cys6 and notably a proline residue after Cys6. The two regions between the pairs of cysteine residues vary considerably in sequence and length between family members and it was originally proposed that this variation might somehow govern the specificity of

functional interaction of proteins containing this motif (Freemont, 1993; Freemont *et al.*, 1991).

RING fingers have enjoyed considerable interest because of their widespread occurrence and involvement in human disease. For example, many of the familial mutations in the breast cancer gene product, BRCA1, are found within the RING domain (Miki *et al.*, 1994). Other RING finger proteins implicated in human disease include the promyelocytic leukemia protein (PML) that is disrupted in acute promyelocytic leukemia (Lavau *et al.*, 1995), the protooncogene Cbl, a negative regulator of growth factor receptor signaling (Bowtell and Langdon, 1995), the melanoma 18 protein (Mel18) (Kanno *et al.*, 1995), and the parkin protein, which is disrupted in autosomal recessive familial juvenile Parkinsonism (Kitada *et al.*, 1998). Additionally, refined sequence analysis has identified subclasses of RING fingers. The tripartite RING motif comprises the RING finger, a second distinct zinc-binding domain, known as the B-box, followed directly by a leucine rich coiled coil (Reddy and Etkin, 1991; Reddy *et al.*, 1992). The RING-H2 family comprises a small number of proteins that contain a histidine residue in the Cys4 position. Other RING variants include the p53 tumor suppressor regulator MDM2 in which Cys3 is substituted with a threonine residue, retinoblastoma binding protein Q1 (RBQ-1) in which His1 is substituted with an asparagine residue and CART1 in which Cys7 is replaced by an aspartic acid residue (Saurin *et al.*, 1996). Outside of these noted differences, the core hydrophobic residues, the sequence and spacing conservations between coordinating cysteine and histidine residues, are well conserved

RING fingers are also unusual because they bind two zinc ions using a unique cross-brace arrangement. Each zinc atom is coordinated with either four cysteines or three cysteines and a histidine (Barlow *et al.*, 1994; Borden *et al.*, 1995). In this system, the first pair of ligands (Cys1 and Cys2) coordinates a zinc ion with the third pair (Cys4 and Cys5)

and the second (Cys3 and His1) and fourth pair (Cys6 and Cys7) coordinates the second zinc atom forming an integrated structural unit (Everett *et al.*, 1993; Schwabe and Klug, 1994). Presently, there have been three structural determinations of isolated RING finger peptides reported. These structures are from the immediate early equine herpes virus (IEEHV) ICP0 protein (Barlow *et al.*, 1994), the human promyelocytic leukemia protein, PML (Borden *et al.*, 1995), and the human immunoglobulin gene recombination enzyme, RAG1 (Bellon *et al.*, 1997). The IEEHV RING finger adopts a ββαβ fold (Barlow *et al.*, 1994) whereas the PML RING finger comprises four β-strand regions, a single turn of 3_{10} helix, and a number of loops and turns (Borden *et al.*, 1995). Both structures use a cross-brace zinc-binding arrangement to bind zinc ions with an inter-zinc distance of approximately 14 Å, but the overall structures are quite dissimilar (Borden, 2000; Freemont, 2000). The crystal structure of RAG1 revealed yet another arrangement with a single domain composed of a RING finger in associated with a Cys2His2 zinc finger to form a unique zinc binuclear cluster in place of a normally mononuclear zinc site in the RING finger (Bellon *et al.*, 1997).

Establishing a definitive function for the RING motif has been difficult. It has been suggested that RING-containing proteins are directly involved in specific DNA binding, mainly due to the existing knowledge of zinc fingers motifs as DNA binding modules (Lovering *et al.*, 1993). However, many RING finger proteins are cytoplasmic, where no inherent DNA binding activity is to be expected (Bordallo *et al.*, 1998; Rothe *et al.*, 1994). RING finger proteins are found in a variety of cellular locations and are reported to mediate a variety of processes including development, oncogenesis, apoptosis and viral replication (Borden and Freemont, 1996). There is, however, little evidence for a RING finger specific function in these proteins. At the molecular level, a myriad of diverse functions including transcription, recombination, RNA processing and peroxisomal biogenesis have been described for RING fingers (Borden, 2000).

A number of studies have evaluated biological activity of the RING finger by either point or deletion mutagenesis analysis. Deletion or mutation of cysteine residues within the RING finger of TRAF2, a member of the TRAF family which transduce signals from members of the TNFR superfamily to the transcription factor NF-kB, abrogate TNF receptor signaling (Takeuchi *et al.*, 1996). Additionally, point mutations of the zinc-binding amino acids in the yeast protein Pas7p RING finger abolishes its activity in peroxisome assembly and zinc binding (Kalish *et al.*, 1995). On the other hand, deletion of the N-terminal RING finger of the bmi-1 oncogene did not alter the ability of this protein to repress homeotic gene transcription (Cohen *et al.*, 1996) and deletion of the RING finger of Drosophila D-IAP did not affect its association with HID and anti-apoptosis function (Vucic *et al.*, 1998). Apart from those point mutants that have targeted the conserved zinc binding ligands, in general, site-specific mutagenesis has failed to prove a critical role for the RING in the specific maintenance of biological function.

Although analysis of the human herpes virus type 1 (HHV1) RING ICP0 protein indicated that surface residues of the central α helix of the RING were important for both nuclear localization and transactivation of gene expression (Barlow *et al.*, 1994; Everett *et al.*, 1995a), a subsequent report indicated the RING finger was not critical for ICP0s localization to the nuclear matrix (O'Rourke *et al.*, 1998). Additionally, surface mutations of the nuclear oocyte protein PwA33, of the newt *Pleurodeles walti*, did not affect the localization of this protein to lampbrush chromosome loops, however, mutation of the zinc coordinating His residue abolished the nuclear localization pattern (Bellini *et al.*, 1995). These conflicting reports likely stem from the plasticity or interchangeability of amino acid residues within proteins or reflect a cooperative dependence on the RING finger and other regions of these proteins to maintain biological function. Although, in general, the integrity of the RING finger domain appears to be essential for function, until the atomic structure of

an entire RING finger protein in combination with a binding partner, either protein or nucleic acid, becomes known, such reports should be viewed with caution. The exact molecular function of the RING finger has been remarkably elusive.

Many RING fingers are found to function in the formation of large protein complexes that may contribute to a diverse range of cellular processes, suggesting RING fingers function in mediating protein–protein interactions (Borden and Freemont, 1996). A variety of RING finger interactions have been described involving both association of different RING finger proteins and association between RING finger and non RING finger related proteins. For example, the BRCA1 protein forms both homodimers and heterodimers through association of RING finger motifs with another RING containing protein, BARD1, which by itself also forms homodimers (Wu *et al.*, 1996). This propensity of RING finger proteins to aggregate has led to difficulties in working with these proteins at the biochemical, structural and biophysical level due to solubility problems.

Additionally, PML forms large macromolecular multiprotein complexes in the nucleus, referred to as PML or ND10 nuclear bodies, which comprise at least five different proteins (Dyck *et al.*, 1994; Koken *et al.*, 1994). Disruption of the RING finger through mutations in conserved cysteine residues results in a loss of PML nuclear bodies (Borden *et al.*, 1995) and this correlates with a loss of growth suppression, transformation suppression (Liu *et al.*, 1995) and apoptotic activities (Borden *et al.*, 1997). It is remarkable that two of the proteins for which RING finger structures have been solved, namely PML and the HHV1 ICP0 ortholog of IEEHV, actually associate. However, the ICP0 RING finger is not required for this association (Everett and Maul, 1994). The ICP0 protein causes redistribution of PML nuclear bodies, releasing their components during herpes virus infection. Although there have been few underlying themes that enable one to assign a particular function to RING fingers, recent novel and highly informative findings indicate

that RING fingers can function in ubiquitination reactions. This seminal finding may tie together previous disparate reports of RING finger proteins interacting with components of the ubiquitination system and provide an increased appreciation for the role of targeted protein proteolysis in the regulation of biological function. For example, PML was reported to be modified by SUMO-1/PIC1/Sentrin (Boddy *et al.*, 1996) and the BRCA1 RING finger was found to interact with BAP1, a ubiquitin hydrolase molecule (Jensen *et al.*, 1998).

Ubiquitination

Ubiquitin is a small 76 amino acid polypeptide that following covalent ligation to target proteins marks them for degradation by the 26S proteosome (Goldberg, 1995). Ubiquitination involves a cascade of enzymatic reactions, the first of which is the activation of the C-terminal Gly residue of ubiquitin in an ATP dependent reaction catalyzed by an E1 ubiquitin-activating enzyme. This reaction forms a thioester linkage of ubiquitin to a Cys residue of the E1 enzyme. The second reaction is catalyzed by the E2 ubiquitin-conjugating enzymes that transfer activated ubiquitin from E1 to an active site Cys residue. The last step in the cascade is the transfer of activated ubiquitin from the E2-ubiquitin intermediate to Lys residues of the substrate or target protein and is catalyzed by the E3 ubiquitin protein ligases (Hershko and Ciechanover, 1998). Generally there is a single E1, but there are many species of E2 and multiple families of E3 or E3 multiprotein complexes. The E3 group is very heterogeneous and appears to be responsible mainly for the selectivity of ubiquitin-protein ligation and thus of protein degradation, however, most of its members are poorly characterized (Karin, 1999).

RING fingers as E3 ubiquitin protein ligases

Currently, it appears that some RING finger motifs are components of protein complexes involved in catalyzing poly-ubiquitination and likely function as E3 ubiquitin protein ligases (Freemont, 2000). It was originally proposed that RING-H2 domains are specific for ubiquitin ligase targeting (Aravind and Koonin, 2000), but classical RING fingers have also been shown to possess ubiquitination activity (Huang *et al.*, 2000; Joazeiro *et al.*, 1999). *In vitro*, a variety of unrelated RING finger proteins have been found to enhance the poly-ubiquitination activity of E2 (Lorick *et al.*, 1999). In a yeast two hybrid screen, using the human E2 ubiquitin conjugating enzyme UbcH5 as target, a new member of the RING family (AO7) was identified. AO7 is able to associate with purified preparations of UbcH5 and can itself act as a substrate for ubiquitination (Lorick *et al.*, 1999). Remarkably, RING finger proteins as GST fusions such as Praja1, NF-X1, kf-1, TRC8, Siah-1 and BRCA1 were all found to bind UbcH5 and support ubiquitination suggesting RING fingers act as E3 ubiquitin ligases (Lorick *et al.*, 1999). The integrity of the RING is required for association with UbcH5 since mutations of conserved zinc-ligating residues or the addition of zinc chelators destroy this activity (Lorick *et al.*, 1999). Additionally, the ubiquitin conjugating enzyme UbcM4, which is necessary for mouse development, interacts with a family of UbcM4 interacting proteins (UIPs) that belong to the RING finger family (Martinez-Noel *et al.*, 1999).

Further support for the RING finger motif in mediating E3 ubiquitin ligase reactions, comes from studies of the protooncogenes Cbl and MDM2. Cbl is a negative regulator of several receptor signaling pathways, including those of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Bowtell and Langdon, 1995; Waterman *et al.*, 1999) and becomes oncogenic *in vivo* as a result of mutation within its RING finger. It has been shown to interact with and recruit the E2 ubiquitin conjugating enzymes UbcH4

and UbcH7 thereby promoting the ubiquitination of activated receptors (Joazeiro *et al.*, 1999; Yokouchi *et al.*, 1999). MDM2, a p53 responsive gene product, is a critical negative regulator of p53 stability and function (Kubbutat *et al.*, 1997). MDM2 binds directly to p53 and promotes its ubiquitination and subsequent degradation in a reaction that is dependent on the presence of the MDM2 variant RING finger (Honda *et al.*, 1997; Honda and Yasuda, 2000).

The current consensus is that the RING finger and surrounding regions probably associate with E2-ubiquitin and provide a favorable environment for the transfer of the ubiquitin from E2 to the targeted lysine (Freemont, 2000; Lorick *et al.*, 1999). The fact that RINGs can participate in ubiquitin transfer to other proteins as well as themselves could represent a novel regulatory mechanism. The RING may target its own or associated proteins for ubiquitin-dependent degradation (Fang *et al.*, 2000). Indeed, the small ubiquitin like polypeptide SUMO-1 allows modification of protein function and localization of both PML and RanGAP (Muller *et al.*, 1998). For PML, the RING is required for binding the ubiquitin conjugating 9 protein (Ubc9), which is specific to SUMO-1, but SUMO-1 modification occurs approximately 400 residues C-terminal to the RING (Duprez *et al.*, 1999).

E3 ubiquitin ligases exist as large macromolecular assemblies (Seol *et al.*, 1999) that critically depend upon the RING finger for their organization and function (Borden, 2000). It remains to be determined, however, whether all or a subset of RING fingers function as E3 ubiquitin ligases. Future research into these RING finger proteins may not only facilitate our understanding of ubiquitination processes in general, but will also likely have major ramifications for the role of ubiquitination in a number of disparate biological functions. For example, recent reports indicate that cIAP2, a documented inhibitor of apoptosis, functions as a ubiquitin-protein ligase and promotes *in vitro* ubiquitination of caspases-3

and -7 (Huang *et al.*, 2000). This provides a plausible scenario for the role of cIAP1s in apoptosis suppression.

Virus Factories

One definitive feature of the poxvirus RING finger proteins is localization to the cytoplasmic viral factories. Poxviral DNA replication occurs in viral factories/virosomes that are discrete regions within the cytoplasm of infected cells. The virosome is a rapidly sedimenting protein-DNA complex that contains newly replicated DNA associated with a variety of virus specified polypeptides, most of which appear to be non-sequence specific DNA-binding proteins (Polisky and Kates, 1976) and do not seem to be incorporated into mature virus particles (Polisky and Kates, 1972; Sarov and Joklik, 1973). Biochemical analysis of these aggregates has identified a number of VV (VV strain Copenhagen unless otherwise designated) associated factory proteins. These include a 36 kDa phosphoprotein variously described as FP11 and polypeptide B which is the product of the H5R gene (Beaud *et al.*, 1995; Nowakowski *et al.*, 1978); the essential B1R kinase (Banham and Smith, 1992); the non-essential DNA ligase encoded by A50R (Beaud, 1995); the recently identified essential single stranded DNA binding phosphoserine protein encoded by the I3L gene (Rochester and Traktman, 1998) and two proteins of 40 kDa and 28 kDa described as FP10 and FP14 respectively (Nowakowski *et al.*, 1978), which remain to be identified. Although the virosome has been inferred to resemble the viral equivalent of a chromatin like matrix (Sarov and Joklik, 1973), the precise relationship between these protein complexes and the large aggregates of viral DNA remain to be elucidated.

Studies with the p28- mutant EV indicated localization of p28 to the virus factories was important for EV virulence. As such it was important to identify critical regions of the poxviral RING finger proteins necessary for factory localization, in order to gain insight into the molecular mechanism of targeting these proteins to the factories and possibly shed

light on their role in virulence. In this study, altered SFV N1R proteins, with deletions and site specific mutations, were transiently expressed in VV infected cells to critically evaluate the role of the RING finger and discern regions of the SFV N1R protein that are required for localization. Deletion mutagenesis implicated a requirement of a small central region of the RING for localization, but the RING motif alone was not sufficient. A chimeric protein, however, in which the RING motif of the herpes simplex virus-1 ICP0 protein replaced the SFV N1R RING motif did localize to virus factories, indicating that the potential specificity for factory localization resided outside the RING motif of N1R. Critical evaluation of an alignment of poxviral N1R orthologs identified a short, highly conserved N-terminal sequence 24-YINIT-28. When this sequence was deleted from N1R, localization was abolished indicating this short region likely played an important role, which is consistent with its high degree of conservation among poxviral RING finger proteins.

While it was not possible to immunoprecipitate the epitope tagged N1R protein from VV infected cells, however, this protein was shown to bind calf-thymus DNA cellulose. Elution from this matrix required 0.5-0.75M NaCl, suggesting that N1R localizes to the factory through an inherent DNA binding activity. Structural prediction analysis suggested that the conserved N-terminal region required for factory localization may form a short β strand and subsequent alignment with several β sheet DNA binding proteins uncovered significant similarity with the ribbon-helix-helix motif family which utilize a short β sheet for specific DNA interaction (Lum and Schildbach, 1999; Suzuki, 1995). Members of this family contain an N-terminal motif consisting of a β-strand followed by two alpha helical regions that are important for both oligomerization and facilitating β-sheet interaction with DNA. This class of proteins includes the Arc and Mnt repressor proteins of Salmonella phage P22 (Knight *et al.*, 1989b; Raumann *et al.*, 1994), the MetJ repressor of *E. coli* (Somers and Phillips, 1992; Somers *et al.*, 1994), the F Factor TraY gene product (Lum and Schildbach, 1999) and the recently described *Pseudomonas aeruginosa* transcriptional activator AlgZ (Baynham *et al.*, 1999).

Characterization of the factory localization of five N1R mutants, each having a single potential β strand residue replaced with alanine (Ala), revealed that Asn 26 was the most important residue for factory localization. In contrast to N1R, which strongly binds DNA and rapidly sediments with the virus factories, SFV-N1RAsn26ΔAla mutant protein was found in the soluble fraction of infected cell lysates and failed to bind DNA cellulose. These results indicate that the N1R RING finger motif may not be central to DNA interactions and that N1R β strand residues particularly Asn 26 are involved in DNA binding and targeting N1R to the virus factories. Furthermore, the recent findings of RING finger motifs as E3 ubiquitin ligases directed the investigation of a role for the poxviral RING finger proteins in protein degradation. Preliminary evidence suggests EV p28 expression may influence protein levels following infection. Although this observation is brief, it is dependent on the presence of the RING finger motif.

MATERIALS AND METHODS

CELL AND VIRUS CULTURE

SFV (strain Kasza), MYX (strain Lausanne), VV (strains WR and IHD-W), BGMK and HeLa cells were provided by Dr. G. McFadden (The John P. Robarts Research Institute, and Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada). The Moscow strain of EV (passage 3; EV-WT) and the recombinant p28- mutant EV virus were generous gifts of Dr. R. Mark L. Buller (Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center, St. Louis, Missouri, USA). The p28- mutant EV virus has been described previously (Senkevich *et al.*, 1994; Senkevich *et al.*, 1995). Tissue culture reagents were obtained from GibcoBRL Inc., Gaithersburg, MD, USA unless otherwise stated. Viruses and cells were cultured with Dulbecco's modified Eagle medium (DMEM: low glucose [contains 1,000 mg/L D-glucose, L-glutamine, pyridoxine hydrochloride, 110 mg/L sodium pyruvate and 3.7 g/L $NaHCO_3$]) supplemented with 10% newborn bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 5 ml/L [100X] GlutaMAX-II to give complete D-MEM, in a humidified 37°C, 7% CO_2 incubator (Sanyo CO_2 Incubator [Model MCO-17A], Caltec Scientific LTD., Vancouver, BC, Canada) except where indicated.

BGMK or HeLa cells were passaged by harvesting confluent monolayers from T150 tissue culture flasks and seeding approximately 20% of the cell population back into flasks. Briefly, the growth media was removed and following brief washing of the cells with prewarmed 37°C SSC (Standard saline citrate; 150 mM NaCl, 15 mM Na citrate, pH 7.2), cells were detached from the monolayer by the addition of 15 mls prewarmed SSC containing 0.25% trypsin. Cells were incubated at 37°C until cells began to round up and detach, then 5 mls of complete D-MEM was added to inhibit trypsin activity and prevent clumping of the cells. The cell suspension was poured into 50 ml conical tubes and

centrifuged at 170g for 3 mins (Beckman GS-15 Centrifuge, Beckman Instruments, Palo Alto, CA, USA). The supernatant was decanted and the pellet resuspended in 10 mls of complete D-MEM. One fifth of the cell suspension was diluted into 20 mls complete D-MEM, added to the flasks, which were then returned to the CO_2 incubator.

Infections of semi confluent BGMK or HeLa cells were performed by removing the growth medium, brief washing with tissue culture phosphate buffered saline (PBS; 0.137 M NaCl, 0.0226 % KH_2PO_4, 0.1185 % Na_2HPO_4, 0.02% KCl [pH 7.4]) and the addition of complete D-MEM containing virus diluted to the desired MOI. For T150 flasks, 6 well tissue culture dishes, and chamber slides, the infection volume used was typically 3 ml, 300 µl and 100 µl respectively. Cells were infected at 37°C for 60 min, with gently rocking every 10 mins, and then 1 complete D-MEM was added. For viral preparations, cells infected with VV were routinely harvested after 2 days and 3 days in the case of EV.

Virus was harvested from infected T150 flasks essentially as described for tissue culture propagation. Infected cell pellets were resuspended in 2 ml ice cold hypotonic swelling buffer (10 mM Tris [pH 8.0], 2 mM $MgCl_2$), incubated on ice for 15 min virus was released by freeze-thawing 3 times using a 37°C water bath and a dry ice-methanol bath. Virus samples were sonicated (Branson Sonifier 450; Branson Ultrasonics, Danbury, CT, USA) on ice at 50% cycle, using an output of 6 for 1 min prior to the addition of complete D-MEM and storage at -70°C.

Viral titrations were performed in duplicate using monolayers of BGMK cells in 12 well plates. After incubation for 2-3 days depending on virus, the medium was removed and virus plaques stained with 1 ml of 1% v/v crystal violet (BDH Chemicals, Toronto, ON, Canada) made in neutral buffer formalin (NBF; 4.07% formaldehyde [pH 7], 0.145 M NaCl, 0.03 M Na_2HPO_4, 0.03 M $NaH_2PO_4.H_2O$). After 10 mins incubation at room temperature (RT), the stain was removed; plates inverted on paper towels and allowed to dry.

TRANSFECTION OF DNA into EUKARYOTIC CELLS

For transfection of QIAGEN purified plasmids into VV infected cells, BGMK cells in 6 well dishes were infected with virus at MOI=3. DNA-lipofectin complexes were formulated by the addition of 5 μg DNA in 100 μl of serum-free D-MEM (SFM) to 100 μl SFM containing 15 μl LipofectACE™ Reagent (GibcoBRL Inc., Gaithersburg, MD, USA) in 3ml round-bottom tubes (No 55.476/013; Sarstedt, Numbrecht, Germany). Following gentle mixing, samples were left at RT for 10 mins, prior to the addition of 0.8 mls of SFM. The lipofectin DNA mixture was gently layered onto BGMK cells that were washed to remove serum, once with PBS and three times with 1 ml SFM. Cells were then incubated for 5 hours, upon which 2.5 mls of complete D-MEM was added to the wells and incubation continued overnight. For transfections of cells cultured on chamber slides for immunofluorescence, the procedure was identical except that volumes were reduced to one fifth. For western blot analysis, cells were trypsinized, harvested by centrifugation at 170g for 5 mins and resuspended in 5 x SDS-PAGE loading buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromophenol blue). Samples were heated at 100°C for 10 mins and then loaded on 12.5% SDS-PAGE gels or stored at -20°C

For the construction of recombinant VV's expressing SFV N1R protein and SFV N1RAsn26ΔAla mutant protein, BGMK cells were infected with VV strain IHDW (MOI=2) followed by transfection of plasmid pMSN1 or pMSN1Asn26ΔAla at 2 hours post infection. After 48 hours, virus was harvested and used to infect huTK- 143 cells. Recombinant VV-N1R and TK^- recombinant VV-N1RAsn26ΔAla were selected by two rounds of growth with 5-bromodeoxyuridine (BrdU [25 μg/ml final conc.]; Sigma-Aldrich, Milwaukee, WI, USA) (Mackett *et al.*, 1984) and plaque purified three times using 1% LMP agarose overlays with screening for beta-galactosidase expression (Chakrabarti *et al.*, 1985). Briefly, well-isolated recombinant blue plaques were isolated by inserting the tip of a

sterile cotton-plugged Pasteur pipette through the agarose to the plaque, aspiration of the agarose plug into the pipette and transferal of the agarose plug to D-MEM. Virus was released by three freeze-thaw cycles and sonication on ice. Following amplification of recombinant plaque isolates, the purity of the recombinant VV's were evaluated by histochemical analysis of virus infected cells. Recombinant VV infected cells (6-well dishes) were fixed for 10 mins with 1 ml NBF, washed with PBS and overlaid with staining solution (1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$ (ferricyanide), 5 mM $K_4Fe(CN)_6$ (ferrocyanide), 2 mM $MgCl_2$ in PBS). Following incubation for 24 hours in a humidified 37°C, 7% CO_2 incubator, the reaction was evaluated by microscopy (ZEISS ID 03 microscope; Carl Zeiss Canada, Don Mills, ON, Canada). Expression of the epitope tagged SFV N1R and SFV N1RAsn26ΔAla mutant proteins was confirmed by western blot analysis of infected cell lysates using Mab H1119.

RECOMBINANT DNA

Restriction and DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA, USA), unless otherwise stated. Oligonucleotide primers (Table 1) were obtained from Canadian Life Technologies, Burlington, ON, Canada. Plasmid pMSN1 has been described previously and contains an epitope tagged SFV N1R ORF under the control of a strong synthetic poxvirus promoter (Upton *et al.*, 1994). C-terminal deletion mutants of the SFV N1R gene were constructed by PCR (Taq DNA polymerase; Stratagene, La Jolla, CA, USA) using a 5' primer (SN2-N) that contains a *Nco* I site immediately upstream of the initiating methionine of the N1R ORF and 3' primers that introduced novel stop codons followed by a *Bam*H I site. N1R mutants ZD1, ZD2, ZD3, ZD4 and ZD5 were obtained from reactions containing 5' primer SN2-N and 3' primers ZD1, ZD3, ZDX, ZDY and ZD4 respectively. PCR reactions (Total 50 µl) carried out in a Minicycler PTC-150-25 (MJ Research Inc., Watertown, MA, USA) contained 5 µl of 10x reaction buffer (500 mM KCl, 200 mM Tris-HCl [pH 8.4], 15 mM $MgSO_4$, 0.1% w/v gelatin), 1 ng pMSN1, 0.5 µl of 25 µM SN2-N primer, 0.5 µl of 25 µM mutant 3' ZD primer, 5 µl of 2 mM dNTP mix, 0.5 µl

(2.5 U/ µl) of recombinant Taq DNA polymerase and double distilled water (ddH_20) to 50 µl. Reactions were overlaid with 30 µl of mineral oil. PCR program ZD1 was employed: 94°C for 2 mins (denaturation), followed by 15 cycles of 92°C for 30 sec (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (extension), a final extension at 72°C for 3 mins and cooling to 4°C.

PCR products were isolated by agarose gel electrophoresis using Tris-acetate buffer (TAE; 0.04 M Tris acetate, 0.001M EDTA). *Hind* III digested Lambda phage DNA, 200 ng per lane, was utilized as molecular markers. DNA was stained with 0.5 µg/ml ethidium bromide in ddH_20 and visualized by a transilluminator (302 nm; UV Transilluminator TM-36, UVP-Ultraviolet Products, San Gabriel, CA). DNA was recovered from ethidium bromide stained agarose gels by excising the desired DNA fragment under long wave UV illumination (366 nm; Mineralight® Lamp UVGL-58, UVP-Ultraviolet Products, San Gabriel, CA) with a sterile scalpel and purification of the DNA using "glass milk". Briefly, 1 ml of NaI solution (90.8% NaI, 1.5% Na_2SO_3) was added to the gel slice in a sterile Eppendorf tube and the gel melted by heating at 50°C. Following complete dissolution of the gel, 2 µl of glass slurry (50% silica 325 mesh "fines" in ddH_2O) was added, mixed well and incubated on ice for 10 mins. Following centrifugation for 30 sec at 14, 000 rpm in a microfuge, the supernatant was discarded and the glass pellet washed 3 times with 500 µl of NEET solution (100 mM NaCl, 1 mM EDTA, 50% ethanol, 10 mM Tris [pH 7.5]). The glass pellet was air-dried, resuspended in 50 µl of ddH_2O and DNA eluted at 50°C for 10 mins.

N1R C-terminal deletion PCR products were ligated to pT7 Blue T-Vector (Amp^R; Novagen, Madison, WI, USA). Ligations, carried out at 16°C overnight, routinely contained 100 ng vector, 50 ng gel purified insert, 4 µl 5X buffer (250 mM Tris-HCl [pH 7.6], 50 mM MgCl2, 5 mM ATP, 5 mM DTT, 25% [w/v] PEG-8000), 1.5 µl 10 mM ATP, 1 µl (1U/ µl) recombinant T4 DNA ligase (GibcoBRL Inc., Gaithersburg, MD, USA), in a total volume of 20 µl. Ligations were transformed into NovaBlue competent cells as described by the

manufacturer (Novagen, Madison, WI, USA) and plated on Luria broth (LB) agar plates containing 50 μg/ml ampicillin (Fluka Biochemika, Buchs, Switzerland) that had been prespread with 35 μl of 50 mg/ml X-gal in DMF and 20 μl of 100 mM IPTG (in water) for blue/white screening of recombinants. Positive clones (white colonies) were identified by colony PCR, essentially as described previously for PCR generation of C-terminal N1R mutants, except 1 μl of overnight LB broth cultures of presumptive recombinants were utilized as template for PCR. PCR positive recombinants were further verified by the isolation and restriction digestion of plasmid DNA.

Plasmid DNA was routinely prepared by alkaline lysis (Birnboim and Doly, 1979). Briefly, 1 ml of overnight LB cultures in eppendorf tubes were centrifuged at 14, 000 rpm for 1 min, the media aspirated and the pellet resuspended in 100 μl of ice cold Solution RAE I (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA. Following incubation at RT for 5 min, 200 μl of freshly made Solution RAE II (0.2 N NaOH, 1% SDS) was added and mixed gently by inversion. Following incubation on ice for 5 min, 150 μl of ice-cold solution RAE III (3M NaOAc, pH 5.2) was added, mixed by inversion and placed on ice. RNase was added to a final concentration of 20 μg/ml and the sample preparation was incubated on ice for 30 mins. Samples were centrifuged for 5 min to remove precipitated material and supernatants transferred to Eppendorf tubes containing 500 μl phenol/chloroform pH 8.0. Samples were vortexed vigorously, placed on ice for 5 mins and the procedure repeated twice. Residual phenol was further removed by transferring the aqueous phase to tubes containing 500 μl chloroform, vortexing and centrifugation for 5 mins. The aqueous phase was carefully transferred to sterile Eppendorf tubes and 2 volumes 100% ethanol (-20°C) added with gently mixing. Following incubation at -20°C for 30 mins, samples were centrifuged for 5 mins, the supernatant quickly decanted, and the white DNA pellet gently washed with 0.5 ml 70% ethanol. The DNA pellet was dried in a 50°C heatblock and resuspended in 20 μl TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). SFV-N1R C-terminal deletion mutants were verified by release of the N1R gene

fragments following restriction enzyme digestion with *Nco* I and *Bam*H I and confirmed by manual DNA sequencing using the Sequenase™ version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio, USA) with T7 promoter primer #369348-1 and U-19mer primer #69819-1 flanking the multiple cloning site, according to the manufacturer's instructions.

For expression of N1R and N1R C-terminal deletions in *E. coli*, the ORFs were subcloned from pT7-blue by digestion with *Nco* I and *Bam*H I and ligated into similarly digested pET19b (Amp^R; Novagen, Madison, WI, USA), transformed into *E. coli* BL21(DE3)pLysS (Cam^R; Novagen, Madison, WI, USA) and selected on LB agar plates containing 50 μg/ml Ampicillin and 34 μg/ml Chloramphenicol (Fluka Biochemika, Buchs, Switzerland).

For transient expression of N1R and N1R mutants in VV infected cells, the N1R ORF and deletion mutations were isolated from pT7-Blue constructs by digestion with *Nco* I and *Bam*H I, and ligated into similarly digested pMIN1 (Upton *et al.*, 1994). pMIN1 contains an epitope tagged IHDW N1R homolog ORF under the control of a strong synthetic late poxvirus promoter, such that replacement of the *Nco* I-*Bam*H I gene fragment with constructed C-terminal SFV N1R mutants, results in a single continuous ORF encoding an epitope tagged N1R protein. Ligations were transformed into competent *E. coli* DH5α as follows; 5 μl of ligation reactions were added to 200 μl *E. coli* DH5α competent cells in prechilled tubes, incubated on ice for 30 mins, followed by heat shock at 42°C for 90 seconds in a water bath and immediate chilling on ice for 2 mins prior to the addition of 800 μl of LB (containing 20 mM glucose) and incubation with vigorous shaking at 37°C to allow expression of antibiotic resistance genes. 200 μl of the transformation mixture was plated onto LB containing 50 μg/ml ampicillin. Plates were inverted and incubated at 37°C.

Recombinants were identified by PCR using MAB-N and respective C-terminal deletion primers (ZD's) and release of the ORFs following digestion with *Nco* I and *Bam*H I. The N1R deletion mutant, which removes 154 amino acids from the N-terminus, was

constructed by PCR using a 5' primer (SZT) with an *Nco* I site for cloning the PCR product into pMSN1 after the MAB epitope tag. Plasmid pUC13-N1R (Upton *et al.*, 1994) was used as template, with primers SZT and SN2-C. The PCR program SZT was employed for amplification (94 °C for 2 mins, then 6 cycles of 92°C for 30 sec, annealing at 45 °C for 1 min, extension at 72°C for 2 mins, followed by 20 cycles of 92°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 2 mins and cooling to 4°C). The agarose gel purified PCR fragment was ligated into pT7Blue T Vector and subcloned into pMIN1 as described for C-terminal N1R mutants.

MAB tagged EV-p28 was constructed by digesting pT7EVN2-28C1 with *Nco* I and *Bam*H I, isolating the EV-p28 gene fragment from agarose and ligating it into similarly digested pMSN1. Plasmid pT7EVN2-28C1, is based on pT7 blue and contains the EVp28 gene sequence flanked by *Nco* I and *Bam*H I restriction sites. Following transformation into *E. coli* DH5α, recombinant clones were identified by PCR using Program SZT and primers VN2-N and VN2-C, which are based on the N and C-terminal gene sequences of the VV IHDW RING finger protein.

The internal deletion of amino acids #45-95 (N1R-ZD7) was constructed by first subcloning the *Sal*I-*Bam*HI gene fragment from pMSN1 into similarly digested pBK-CMV (Stratagene, La Jolla, CA). Full length linear DNA was isolated after a partial *Dra*I digestion, subjected to complete digestion with *Eco*RV and religated. Clones with the correct deletion were isolated and the gene fragment subcloned back into *Sal*I-*Bam*HI digested pMSN1.

The SFV N1R-HSV ICP0 RING fusion gene was constructed by PCR Gene Soeing (Vallejo *et al.*, 1995). This construct contains the gene sequence for a chimeric protein consisting of N1R with its RING finger motif substituted by that of ICP0. Plasmid pSHZ containing the HSV-1 ICP0 gene was generously provided by Dr. Stephen Rice (Department of Microbiology, University of Minnesota, Minneapolis, Minnesota, USA).

Primers N1R-X and HSV-Y were designed to have overlapping complementary base sequence such that annealing of PCR products from PCR reactions of the individual gene sequences would overlap resulting in hybridization and subsequent extension by cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). Individual PCR reactions (total 50 µl) contained 5 µl of 10x reaction buffer (200 mM Tris-HCl [pH 8.8], 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton-X-100, 1000 µg/ml nuclease-free BSA), 0.4 µl dNTPs (25 mM each NTP), 100 ng template, 250 ng each of EVZN and N1R-X or HSV-Y and HSV-C primers and 2.5 U cloned *Pfu* DNA polymerase. For PCR amplification of the ICP0 RING finger gene region sequence and N1R N-terminal sequences, a 2.1 kb fragment released from pSHZ following digested with *Sal* I and *Xho* I and pMNS1 were used as respective templates. PCR program CHI was employed: 94 °C for 2 mins, followed by 25 cycles of 92°C for 30 sec, 69°C for 30 sec, 72°C for 1 min and then cooling to 4°C. The PCR products from individual reactions were isolated from agarose and combined to a final concentration of 10 ng/100 µl PCR reaction volume and the full length chimeric gene amplified using EVZN and HSV-C primers. The PCR product was digested with *Nco* I and *Bam*H I, agarose gel purified and ligated into similarly digested pMSN1. Recombinant clones were identified by PCR using primers EVZN and HSV-C and restriction enzyme digestion with *Eco*R V or *Nde* I digestion. Following automated DNA sequencing (Applied Biosystems 373-A automated sequencers; Dr. Ben F. Koop Laboratory, Department of Biology, University of Victoria, British Columbia, Canada), it was found that primers N1R-X and HSV-Y, were incorrectly designed resulting in a gene fusion between N1R gene sequences and HSV ICP0 gene sequences beginning with those encoding Cys 2 of the HSV ICP0 RING finger. In order to restore the gene sequences encoding the intervening amino acid region between Cys 1 and Cys 2 of the ICP0 RING finger, site directed insertional mutagenesis was employed using the QuikChange™ (Braman *et al.*, 1996) Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

Primer SHCC1 and its complement SHCC2 were designed to incorporate an *Apa*L I restriction site. PCR reactions (50 µl total) contained 5 µl of 10x reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.8), 20 mM $MgSO_4$, 1% Triton-X-100, 1 mg/ml nuclease-free BSA), 10 ng pMNS1HSV fusion, 125 ng each of designed mutagenic primer SHCC1 and its complement SHCC2, 1 µl of dNTP mix, double distilled water (ddH20) to 49 µl and 1 µl of Pfu DNA polymerase (2.5 U). PCR was carried out using the MUTG PCR program (denaturation at 95°C for 30 sec followed by 18 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min and extension at 68°C for 16 mins. Reactions were cooled to 4°C prior to addition of 1 µl of restriction enzyme *Dpn* I (10U/µl) to each amplification reaction, gentle mixing by pipetting, microcentrifugation for 1 minute at 14, 000 rpm and incubation at 37°C for 1.5 hours (to digest the parental methylated DNA). 1 µl of the *Dpn* I-treated DNA was added to 50 µl Epicurian Coli® XL1-Blue supercompetent cells Stratagene, La Jolla, CA, USA), mixed by gently swirling and placed on ice. 30 mins later, the transformation reactions were heat-pulsed for 45 seconds in a 42°C water path and immediately placed on ice for 2 mins. 0.5 ml of preheated 42°C NZY+ broth (1% casein hydrolysate, 0.5% yeast extract, 0.5% NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 20 mM glucose) was added to each transformation mixture and incubated at 37°C for 1 hour with vigorous shaking. 250 µl of each transformation mixture was plated on LB agar plates containing 50 µg/ml ampicillin. Plates were incubated at 37°C for 16-20 hours. Mutants were identified by the presence of an additional *Apa*L I restriction site compared to the pMNS1HSV fusion construct and confirmed by automated DNA sequencing. The construct, which replaces amino acids (aa) 172-234 of the N1R protein with aa 116-171 of ICP0, was utilized for transfection into VV-infected BGMK cells.

Site directed and deletion mutagenesis of SFV N1R was similarly carried out using the Quikchange™ Site-Directed mutagenesis kit with plasmid pMSN1 as template and

designed primers (see Table 1) incorporating (in order to facilitate screening) novel restriction enzyme sites or destroying restriction enzyme sites within the N1R gene sequence. Plasmids N1R-mFG190/191LR, N1R-m193L, N1R-mI202N encoding site specific mutations within the RING finger of N1R were respectively constructed using primers ZNG191-A, ZNL193-A, ZNI202A and their complements. Plasmid N1R-d6 which deletes SFV N1R amino acids 24-28 was constructed using primer ZNDEL-A and its complement ZNDEL-B. Site specific Ala mutants of SFV N1R amino acids 24-28 were constructed using respective primers Y24AA, I25AA, N26AA, I27AA, T28AA incorporating a *Bst*U1 site and their complements. The annealing temperature employed to obtain successful PCR amplification products for N1R Ala specific mutants I25, N26, and T28 was 50°C. Constructs were verified by automated DNA sequencing.

For transfection of constructed mutants in VV infected BGMK cells, high quality plasmid midipreps were purified using QIAGEN tip 100 columns (Qiagen Inc., Chatsworth, CA, USA) as directed by the manufacturer. The concentration of DNA was quantitated by measuring the absorbance of a 1:100 dilution of each preparation at 280 nm using the Warburg program (Beckman Du -65 Spectrophotometer; Beckman Instruments, Columbia, MD, USA).

For the analysis of MYX RING finger gene mRNA expression by Northern blotting techniques, samples of 10^6 BGMK cells were mock infected or infected with MYX virus (MOI=10). At 1, 2, 3 and 4 hours post infection, cells were harvested using SSC containing trypsin and washed in PBS. Total RNA was isolated and purified using the RNeasy™ Total RNA kit (Qiagen Inc., Chatsworth, CA) as directed by the manufacturer with the use of QIAshredder columns to release RNA. The recovered RNA was stored at -70°C prior to analysis.

Northern blotting was preformed as follows. RNA was prepared for separation on a 1% agarose gel by incubating 10 µl of isolated RNA with 2.5 µl of 10x MOPS, 3.5 µl

formaldehyde (37%), and 9 µl of deionised formamide for 15 mins at 55°C prior to cooling on ice and the addition of 2.5 µl of loading dye (50% glycerol, I mM EDTA, 0.05% bromophenol blue). 4 µl of 0.16-1.77Kb RNA ladder (1 µg/µl RNA; Gibco BRL) was used as molecular weight markers. Samples were electrophoresed at 100 V for 3 hours on a 1% agarose gel containing 20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, 0.666% formaldehyde, 0.5 µg/ml ethidium bromide, using MOPS running buffer (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, [pH 7.0]). The gel was photographed with UV illumination. The relative distance migrated was measured by placing a ruler alongside the gel.

RNA was transferred to a BioTrace HP (Charged, modified polysulfone; Gelman Sciences, Ann Arbor, Michigan, USA) membrane by capillary action using a blot reservoir containing 10x SSC and sandwiching the gel and membrane between Whatman filter paper sheets, paper towels and a 1 kg weight. Blotting was allowed to proceed overnight at RT. A DNA probe was prepared by digesting plasmid p19Mn2-1, containing the gene sequence for the MYX RING finger protein, with *Nco* I and *Bam*H I and gel purification of the approximately 750 bp gene fragment and radiolabeling with ^{32}P-dCTP (DuPont NEN; Boston, MA, USA) as follows: 50 ng (20 µl) of DNA in TE buffer was denatured by heating for 3 mins at 100°C. Following incubation on ice for 2 mins, 10 µl of 5x Klenow buffer (50 mM Tris-HCl, 25 mM $MgCl_2$, 37.5 mM DTT), 14 µl ddH_20, 50 µCi (5 µl) of labeling nucleotide and 1 µl (5 U) of recombinant DNA polymerase I large fragment (Klenow) was added and the labeling reaction incubated at 37°C for 1 hour upon which the reaction was heated to 95°C for 2 mins to denature the polymerase and then immediately cooled on ice.

The BioTrace HP membrane was placed according to manufacturers instructions in hybridization buffer (1% BSA, 1 mM EDTA, 6% NaH_2PO_4, 7% SDS [pH 7.2]) and prewarmed to 65°C in a 38 x 300 mm hybridization tube. After 15 minutes blocking in a rotating 65°C hybridization incubator (Lab-Line Instruments, Melrose Park, IL, USA), the

hybridization buffer was decanted and fresh hybridization buffer containing the radioactively labeled probe added back. Following overnight hybridization, the buffer was removed to a radioactive container and replaced with 150 ml wash solution 1 (0.005% BSA, 1 mM EDTA, 0.48% NaH_2PO_4, 5% SDS [pH 7.2]). Following 3 washes for 20 mins each at 65°C, the membrane was further washed three times in wash solution 2 (1 mM EDTA, 0.24% NaH_2PO_4, 1% SDS [pH 7.2]). Following discard of the last wash, the membrane was removed, sealed with saran wrap prior to film (BIOMAX™ MR Kodax Scientific Imaging film; Eastman Kodak, Rochester, NY, USA) exposure and incubated at -70°C until development.

Cloning of C-terminal deletions for insertion into pMSN1		
MAB-N	5'*TCGACATGGCGACTGACATTGATATGCTAATTGACCTCGGTGC*	(contains 5'*Sal* I site)
SN2-N	5'*CCCCATGGATCACAACGTTAAAATAT*	(creates *Nco* I site)
SN2-C	5'AAGGATCCTTGTATTTATAGGAC	(creates *BamH* I site)
ZD1	5'*GGATCCTCA*CCTACATACGGGACATTT	(creates *BamH* I site)
ZD3	5'*GGATCCTCA*TTTGTTATTTTGTTTTTT	(creates *BamH* I site)
ZDX	5'GGTTTTTT*GGATCCTCA*ACAACACTCGATGCAGAATACGTGA	(creates *BamH* I site)
ZDY	5'GGTTTTTT*GGATCCTCA*CTCGATGCAGAATACGTGA	(creates *BamH* I site)
ZD4	5'*GGATCCTCA*TACGTGATTGCAGTGAGA	(creates *BamH* I site)
SZT	5'*CCATGGA*TATATTATACAAAGCCATC	(creates *Nco* I site)
Cloning of C-terminal deletions for insertion into pET-19b		
SN2-N	5'*CCCCATGGATCACAACGTTAAAATAT*	(creates *Nco* I site)
ZD1	5'GGATCCTCACCTACATACGGGACATTT	(creates *BamH* I site)
ZD3	5'*GGATCCTCA*TTTGTTATTTTGTTTTTT	(creates *BamH* I site)
ZDX	5'GGTTTTTT*GGATCCTCA*ACAACACTCGATGCAGAATACGTGA	(creates *BamH* I site)
ZDY	5'GGTTTTTT*GGATCCTCA*CTCGATGCAGAATACGTGA	(creates *BamH* I site)
ZD4	5'*GGATCCTCA*TACGTGATTGCAGTGAGA	(creates *BamH* I site)
Site directed mutagenesis of N1R		
ZNG191-A	5'GAATAGCTTTTTA*CGCGT*TTTATCTCACTG	(creates *Mlu* I site)
ZNG191-B	5'CAGTGAGATAAAA*CGCGT*AAAAAGCTATTC	(creates *Mlu* I site)
ZNL193-A	5'GCTTTTTTGGTGT*GAATTC*TCACTGTAATC	(creates *EcoR* I site)
ZNL193-B	5'GATTACAGTGAG*AATTC*ACACCAAAAAAGC	(creates *EcoR* I site)
ZNI202-A	5'CTGTAATCACATATTTTGCAACGAGTGTATAGATAG	(destroys *SfaN* I site)
ZNI202-B	5'CTATCTATACACTCGTTGCAAAATATGTGATTACAG	(destroys *SfaN* I site)
ZNG191-A	5'GAATAGCTTTTTA*CGCGT*TTTATCTCACTG	(creates *Mlu* I site)
ZNG191-B	5'CAGTGAGATAAAA*CGCGT*AAAAAGCTATTC	(creates *Mlu* I site)
ZNL193-A	5'GCTTTTTTGGTGT*GAATTC*TCACTGTAATC	(creates *EcoR* I site)
ZNL193-B	5'GATTACAGTGAG*AATTC*ACACCAAAAAAGC	(creates *EcoR* I site)
ZNI202-A	5'CTGTAATCACATATTTTGCAACGAGTGTATAGATAG	(destroys *SfaN* I site)
ZNI202-B	5'CTATCTATACACTCGTTGCAAAATATGTGATTACAG	(destroys *SfaN* I site)

Table 1. List of oligonucleotide primers utilized in cloning procedures (chapter 1)

ZNDEL-A	5'CTAAGATCTAATCACCGGTTATGTAACCCTATG	(creates *BsrF* I site)
ZNDEL-B	5'CATAGGGTTACATAACCGGTGATTAGATCTTAG	(creates *BsrF* I site)
Y24AA	5'CTAAGATCTAATCACGCGATTAACATAACTCG	(creates *BstU* I site)
Y24AB	5'CGAGTTATGTTAATCGCGTGATTAGATCTTAG	(creates *BstU* I site)
I25AA	5'CTAAGATCTAATCATTACGCGAACATAACTCGATTATG	(creates *BstU* I site)
I25AB	5'CATAATCGAGTTATGTTCGCGTAATGATTAGATCTTAG	(creates *BstU* I site)
N26AA	5'GATCTAATCATTATATCGCGATAACTCGATTATG	(creates *BstU* I site)
N26AB	5'CATAATCGAGTTATCGCGATATAATGATTAGATC	(creates *BstU* I site)
I27AA	5'CTAATCATTATATTAACGCGACTCGATTATGTAACCC	(creates *BstU* I site)
I27AB	5'GGGTTACATAATCGAGTCGCGTTAATATAATGATTAG	(creates *BstU* I site)
T28AA	5'CATTATATTAACATAGCGCGATTATGTAACCCTATG	(creates *BstU* I site)
T28AB	5'CATAGGGTTACATAATCGCGCTATGTTAATATAATG	(creates *BstU* I site)

Gene Soeing

EVZ-N	5'CCCCAAAAGCTAGCGACTGACATTGATATGCAT	(creates *Nhe* I site)
N1R-X	5'GGGCGCGATCTCATCCGTGCATTCCTCGCCTTTATACC	
HSV-Y	5'CAGGTATAAAGGCGAGGAATGCACGGATGAGATCGCGCCC	
HSV-C	5'GTGGGATCCTACCCGCTGGGCGTCACGCCC	(creates *BamH* I site)

Insertion of wild type gene sequence from Cys1 to Cys 2 of HHV1 ICP0 in Gene Soeing construct by site directed mutagenesis.

SHCC1	5'GGTATAAAGGCGAGGAATGCGCCGTGTGCACGGATGAGATCGCGCCCC	(creates *ApaL* I site)
SHCC2	5'GGGGCGCGATCTCATCCGTGCACACGGCGCATTCCTCGCCTTTATACC	(creates *ApaL* I site)

Epitope tagging of EV-p28

VN2-N	5'CCCCATGGAATTCGATCCTGCC	(creates *Nco* I site)
VN2-C	5'AAGGATCCTTAGTTAACTAGCTTATAGAA	(creates *BamH* I site)

Table 1 (continued). List of oligonucleotide primers utilized in cloning .

PROTEIN EXPRESSION

SFV N1R and SFV N1R C-terminal deletion mutants were expressed in *E. coli* BL21(DE3)pLysS using an inducible T7 expression system and recovered as insoluble inclusion bodies. Briefly, isolated colonies of pET19b-N1R BL21(DE3) pLysS and respective C-terminal N1R deletion mutants were grown to an OD_{600} of 0.6 in 3 mls of LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. 1 ml of the above starter culture was inoculated into 30 mls of the same growth medium, grown to an OD_{600} of 0.5 (approx. 2.5 hours) and IPTG added to a final concentration of 1 mM. 3 hours later, cells were recovered by centrifugation at 7, 000 g for 5 mins and inclusion bodies recovered (Harlow and Lane, 1988). The cell pellet was resuspended in 2.5 mls Buffer-1 (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0). Lysozyme was added to a final concentration of 1 mg/ml and the samples incubated at RT for 20 mins. Spheroplasts were recovered by centrifugation at 3,500 g for 10 mins at 4°C, resuspended in 5 mls of ice cold Buffer-2 (100 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0 and incubated on ice for 10 mins with occasional mixing. $MgCl_2$ and DNase I were added to a final concentration of 8 mM and 10 μg/ml respectively. Samples were incubated at 4°C with occasionally mixing until the high viscosity disappeared. Inclusion bodies were recovered by centrifugation at 13,800 g for 10 mins. Pellets were washed twice by resuspension in Buffer-3 (1% NP-40, 100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0). Following a final wash in Buffer-1, inclusion bodies were resuspended in 500 μl Buffer-1 and stored at -20°C.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970) using a minigel apparatus (Mini Protean II, Biorad, Richmond, CA). Proteins were stacked using a 4% stacking gel and were typically separated on 12.5% gels, unless otherwise stated. Gels were run at 200 V (BIO-RAD Power Pac 300; Biorad, Richmond, CA) in SDS-PAGE electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS [pH 8.3]). Full length and mutant N1R proteins

were solubilized by the addition of 5x SDS-PAGE sample buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromophenol blue) and incubation at 100°C for 5-10 mins. Following separated on 12% SDS-PAGE, proteins were visualized by staining with Coomassie Blue R-250 solution (0.3% Coomassie blue, 45% methanol, 10% acetic acid) or for zinc binding analysis (performed by Dr. Leslie Schiff, Department of Microbiology, University of Minnesota, Minneapolis, Minnesota, USA), electroblotted to nitrocellulose (see below) and probed with $^{65}ZnCl_2$ as previously described (Schiff *et al.*, 1988).

For the generation of antisera to N1R, *E. coli* expressed N1R protein was separated on a 12.5% SDS-PAGE Midi gel using a Model SE 650 electrophoresis tank apparatus (Hoefer Scientific Instruments, San Francisco, USA). Broad range prestained protein molecular weight markers (Biorad, Richmond, CA) served as molecular weight standards. Following staining with Coomassie blue and destaining with 35% methanol, 10% acetic acid, the N1R protein band was carefully excised using a sterile scalpel. The gel fragment was placed in an Eppendorf tube and dried with heating at 37 °C in a Vacufuge (RC 10.10 Jouan; Canberra-Packard Canada Ltd., Toronto, ON, Canada) overnight. The resulting desiccated gel fragment was ground to a fine powder using a mortar and pestle, mixed with complete Freund's adjuvant and utilized to immunize mice. Immunization of mice and production of hybridomas and ascites fluid (conducted by Robert P. Beecroft, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada) was performed (Kohler and Milstein, 1975), with modifications as described (Stebeck *et al.*, 1996).

Virus infected, uninfected and transfected BGMK cell lysates were prepared for SDS-PAGE by resuspending approximately 1 x 10^6 cells in 100 µl hot 10% SDS. 20 µl aliquots of lysates were mixed with 5 µl of 5X SDS-PAGE sample buffer and heated at 100°C prior to loading samples (20 µl) per well. Broad range prestained protein molecular weight markers (Biorad, Richmond, CA) served as molecular weight standards and also as

positive controls for electrophoretic protein transfer to membranes. Electrophoretic transfer of proteins from SDS-PAGE minigels onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes (both from Gelman Sciences, Ann Arbor, Michigan, USA) was carried out using a semi-dry blotting apparatus (Tyler Research Instruments, Edmonton, Alberta, Canada). Nitrocellulose membranes were wet by direct immersion in semi-dry transfer buffer (48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS, 20% methanol [pH 9.2]). PVDF membranes were first wet in 100% methanol, with agitation for 5 mins, followed by the gradual addition of semidry-transfer buffer to equilibrate the blot with the transfer buffer. SDS-gels were allowed to equilibrate with semi-dry transfer buffer for at least 5 mins prior to assembly of the transfer apparatus. The membrane and gel were sandwiched between 12 Whatman filter papers (cut to the same size as the gel and membrane) that were wet with semi -dry transfer buffer. Transfer was carried out at 200 mA for 2 hours.

IMMUNOLOGICAL METHODS

Mouse monoclonal antibody (mAb) H1119 (Goodwin Institute for Cancer Research, Plantation, Fl.) recognizes an epitope in a 12 amino acid sequence of the herpes simplex virus type 1 ICP27 protein. The gene sequence for this epitope is fused to the N-terminus of the N1R gene in plasmid pMSN1 and transfection of pMSN1 and constructed mutants into VV infected cells were performed as previously described (Upton *et al.*, 1994). Protein samples separated by SDS-PAGE and transferred to PVDF membrane (Gelman Sciences, Ann Arbor, MI, USA) were blocked with 3% BSA (Fluka Biochemika) in either Tris-buffered saline (100 mM Tris, 4.5% NaCl [pH 7.5]) or PBS (0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 0.02% KCl, [pH 7.4]) overnight, prior to incubation with mAb H1119 (1: 2000). Typically PBS was used when utilizing peroxidase conjugated secondary antibodies and TBS with use of alkaline phophatase conjugated secondary antibodies. Following incubation with mAb H1119 for 4 hours, membranes were washed three times for 5 mins each with either TBS or PBS-Tween (0.8% NaCl, 0.02% KH_2PO_4, 0.115%

Na_2HPO_4, 0.02% KCl, 0.05% Tween-20 [pH 7.4]). Blots were incubated with either alkaline phosphatase conjugated goat-anti-mouse IgG (Bio-Rad; 1:2, 000) or horseradish peroxidase conjugated goat anti-mouse IgG (1:5000) (Caltag laboratories Inc., Burlingame, CA, USA) in 3% BSA for 2 hours. Following washing of the membranes as above, bound alkaline phosphatase or horseradish peroxidase conjugated secondary antibodies were detected by the Immun-Blot alkaline phosphatase assay (Bio-Rad Laboratories, Hercules, CA) or Supersignal® chemiluminescent substrate (Pierce Chemical, Rockford, IL, USA) respectively, as described by the manufacturers.

Immunoprecipitation (IP) analysis of recombinant VV expressed SFV N1R using mAb H1119 was carried out using a variety of methods (Ciccone *et al.*, 1988; de Gunzburg *et al.*, 1989; Rothe *et al.*, 1994). Samples of 6 x 10^6 BGMK cells in 100 mm tissue culture dishes were either mock infected or infected with VV or VV-N1R (MOI=10). At 5 hours post infection, cells were washed twice with tissue culture PBS and 3 mls of Dulbecco's Modified Eagle Medium, high glucose labeling medium (contains 4, 500 mg/L D-glucose, pyridoxine hydrochloride, but no L-glutamine, L-methionine, or L-cysteine; GibcoBRL) containing 100 µCi ^{35}S (NEG-072 EXPRE^{35}S^{35}S [^{35}S] protein labeling mix; Dupont NEN Research products, Boston, MA, USA) added to the cells. After 30 mins, the label was removed and replaced with 3 mls of labeling medium. 45 mins later, cells were detached from the dishes using SSC supplemented with trypsin and transferred to screw capped microfuge tubes. Following one wash with PBS, cell pellets were transferred to the 4°C coldroom. Cell lysis and formation of immunoprecipitates were carried out as follows:

Method A (Ciccone *et al.*, 1988): Cells were gently resuspended and lysed for 30 mins in 1 ml NP-40 lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 1 mM PMSF, 50 mM Tris pH 7.2). Each individual sample was divided into two aliquots of 500 µl. 4 µl of either mAb H1119 or mAb 5013 (mouse anti beta-galactosidase [*E. coli*] clone BG-01; Monosan/Cedarlane laboratories, Hornby, ON,

Canada; used as a positive IP control) was added to individual 500 µl samples and incubated overnight on a rocker platform (Bellco Biotechnology, Vineland, New Jersey, USA). 4 µl of ImmunoPure® rabbit anti-mouse IgG Fc unconjugated secondary antibody No.31194 (Pierce Chemical) was then added and incubation with rocking at 4°C continued for 5 hours. Immune complexes were collected by the addition of 30 µl of Immunoprecipitin (Formalin-fixed Staph A cells; GibcoBRL) prepared as follows: cells were centrifuged at 3, 000 rpm in a microfuge for 10 mins and resuspended in an equal volume of PBS (0.15 M NaCl, 0.01 M Na_2HPO_4 [pH 7.2]), containing 10% w/v β-mercaptoethanol and 3% w/v SDS. Following incubation at 95°C for 30 mins, cells were pelleted as above and resuspended in an equal volume of lysis buffer.

Following incubation for 30 mins at 4°C, the immunoprecipitate was recovered by centrifugation at 4, 000 rpm for 15 mins in a microfuge, gently resuspended in 500 µl lysis buffer and transferred to fresh screw capped microfuge tubes. The immunoprecipitate was washed 3 times with 500 µl of lysis buffer and following a final wash in buffer TN (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.2]), the immunoprecipitate pellet was resuspended in 75 µl of 2x SDS-PAGE sample buffer containing 5% fresh β–mercaptoethanol and 8 M urea. Samples were boiled at 100°C for 10 mins. Staph A cells were pelleted by centrifugation at 14, 000 rpm in a microfuge for 5 mins and samples of the supernatant (35 µl) loaded and run on 10% SDS-PAGE gels. For autoradiography of 35^S-labeled proteins, SDS-PAGE gels were incubated in 5% glycerol for 2 hours prior to transfer onto 3 mm Whatman paper, gel drying (Jouan GF-10) at 60°C and detection by autoradiography.

Method B (Rothe *et al.*, 1994): Cells were lysed with 1 ml buffer-1 (0.1% NP-40, 50 mM Hepes [pH 7.2], 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF) for 30 mins at 4°C. Antibody incubations and procedures were as described for method 1, except that

Immunoprecipitin was resuspended in buffer-1. Washes of the immunoprecipitate were carried out using 500 µl of buffer-1.

Method C (de Gunzburg *et al.*, 1989): Cells were lysed in HEPES buffer (50 mM Hepes [pH 7.4], 0.1 M NaCl, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF). Antibody incubations were as described for method-1. Immunopreciptitin was prepared in Hepes buffer. Immunoprecipitin-immune complexes were washed twice in RIPA buffer (10 mM Tris [pH 7.5], 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl) and twice using RIPA without NaCl, prior to taking up in 75 µl of 2x SDS-PAGE sample buffer containing 5 % fresh β-mercaptoethanol and 8 M urea.

For immunofluorescence and confocal microscopy analysis of the localization of epitope tagged N1R and N1R mutants using mAb H1119 within VV-infected BGMK cells, BGMK cell cultures were grown in 8 chamber slides (SuperCell, Fisher Scientific, Pittsburgh, PA), infected with VV strain WR (MOI=5) and transfected (LipofectAce, GibcoBRL) with 1 µg of vector, purified on QIAGEN columns (Chatsworth, CA), at 2 hr post infection. After 18 hr, cells were fixed (4% paraformaldehyde in PBS) for 30 min at 4°C, rinsed three times with tissue culture PBS and blocked at RT for 30 mins with 5% Normal Lamb serum, 0.01% Tween 20 in PBS. Following rinsing with PBS, cells were incubated with primary antibody (H1119; 1:200) in blocking buffer overnight at 4°C. Preparations were then rinsed in PBS, and incubated with FITC conjugated goat anti-mouse antibody (Biodesign International, Saco, Maine, USA) for 2 hours at RT. For screening hybridoma supernatants for the presence of reactive antibody to SFV expressed N1R following infection of BGMK cells, cells were fixed with -20°C methanol for 10 mins, washed with PBS and hybridoma supernatants (used neat) incubated for 2 hours at RT, prior to incubation with FITC conjugated goat anti-mouse antibody for 1 hour and

immunofluorescence analysis. For DNA visualization studies of VV transfected cells, slides were also stained with bisBenzimide Hoechst 33342 (Sigma Chemical, St. Louis, MO) at 500 ng/ml in PBS for 30 min. After rinsing, coverslips were mounted with Slow Fade (Molecular Probes, Eugene, OR) and viewed with epifluorescence or with confocal laser scanning microscopy. Confocal images were collected on a Zeiss LSM 410 inverted microscope. Series images, were collected with 4X line averaging, scanning at 8 sec per frame, images were 512x512 pixels. Projections of series, or portions of series were use to make images.

DNA CELLULOSE CHROMATOGRAPHY

Extract preparation and DNA cellulose chromatography were modified from the methods of Alberts and Herrick (Alberts and Herrick, 1971). Initially the isolation of SFV N1R protein from VV infected BGMK cells for DNA cellulose chromatography involved infection of 10^7 BGMK cells with recombinant VV-N1R (MOI=3) and harvesting the cells at 24 hours post infection. Briefly, cells were trypsinized in SSC, recovered by centrifugation (670 g), washed twice in PBS, and resuspended in 2 mls of a modified extract buffer; NETP (1 mM EDTA, 50 mM Tris-HCl [pH 8.0], 3 mM β-mercaptoethanol). Following dounce homogenization, the extract was centrifuged as above and the presence of N1R within the pellet or supernatant fractions determined by western blotting using mAb H1119.

Attempts to release N1R from the insoluble pellet fraction involved the addition of increasing concentrations of urea and NaCl to samples of the pellet resuspended in NETP, incubating on ice for 30 mins and determination of the presence or absence of N1R within the supernatant or pellet fraction after centrifugation in a microfuge (14, 000 rpm, 5 mins) and western blotting. Following release of N1R with NETP containing 0.6M NaCl, PEG 6000 (Fisher Biotech, Pittsburgh, PA, USA) was added to the extract to a final concentration of 10%. After incubation for 30 mins at 4°C and centrifuged at 5, 000 rpm for 10 min,

western blot analysis of the resulting pellet and supernatant fractions indicated the addition of PEG 6000 destroyed the ability to detect the presence of the mAb epitope. An alternative strategy was then employed to harvest VV-N1R infected cells and shear DNA in the sample using a fine gauge needle.

10^8 BGMK cells were infected with the recombinant VV-N1R (MOI=3). After 4 hours, the infected cells were transferred to 32°C in an attempt to promote correct folding of the recombinant protein, and harvested 24 hr post infection. Cells were trypsinized in SSC (150 mM NaCl, 15 mM Na citrate, pH 7.2), recovered by centrifugation (670 g, 5 min), washed twice in PBS, resuspended in a hypotonic swelling buffer (10 mM Tris pH 8.0, 2 mM $MgCl_2$) and lysed using a dounce homogenizer. Following centrifugation (800g, 5 min) the N1R protein was extracted from the pellet using a high salt buffer (50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 3 mM 2-mercaptoethanol, 1 mM EDTA, 10 μM $ZnCl_2$, 1 mM PMSF) for 30 min on ice. Soluble N1R was recovered in the supernatant following centrifugation (1,000 g, 15 min).

For DNA cellulose chromatography, the soluble N1R extract was desalted using a 10 ml KwikSep™ polyacrylamide 6000 desalting column (Pierce Chemical, Rockford, IL) into buffer-1 (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 3 mM 2-mercaptoethanol, 1 mM PMSF). 10 ml KwikSep™ polyacrylamide 6000 desalting column (Pierce Chemical, Rockford, IL) were prepared for desalting by equilibrated with 50 mls of 0.2 M ammonium bicarbonate, pH 8.0 followed by 50 mls of buffer-1 prior to sample loading. Double-stranded and single-stranded DNA cellulose (Pharmacia Biotech, Piscataway, NJ) was prepared in buffer-1. Protein samples in the same buffer were applied to 1 ml columns at a flow rate of 1 ml/hour, and recycled through the column twice. The columns were washed with 6 ml of buffer-1, and then eluted stepwise (1 ml steps) with buffer-1 containing 0.1 M, 0.25 M, 0.5 M, 0.75 M, 1.0 M, and 1.5 M NaCl. Samples were analyzed by western blotting after SDS-PAGE and electroblotting to nitrocellulose.

For the isolation of VV-N1RAsn26ΔAla containing extracts for DNA cellulose chromatography, the above procedure was slightly modified. 10^8 BGMK cells were infected with the recombinant VV-N1RAsn26ΔAla or VV-N1R as positive control for DNA binding (both at MOI=3). Following harvest of the infected BGMK cells and resuspension in swelling buffer (10 mM Tris pH 8.0, 2 mM $MgCl_2$), $ZnCl_2$ was added to a final concentration of 10 μM and cells were lysed using a dounce homogenizer. Following centrifugation (800g, 5 min) mutant N1RAsn26ΔAla protein was detected by western blot with mAb H1119 within the supernatant, whereas N1R remained tightly associated with the virosome pellet fraction and required extraction with salt buffer (50 mM Tris-HCl pH 8.0, 0.6 M NaCl, 3 mM 2-mercaptoethanol, 1 mM PMSF) for 30 min on ice. Viscous DNA in the sample was sheared by passing the lysate through a 26G1/2 PrecisionGlide needle (Becton Dickinson). Soluble N1R was recovered in the supernatant following centrifugation (2, 500 rpm, 15 min).

For DNA cellulose chromatography, both the salt extracted N1R and supernatant containing N1RAsn26ΔAla extracts were passed through a 10 ml KwikSep™ polyacrylamide 6000 desalting column (Pierce) into buffer-X (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 3 mM 2-mercaptoethanol, 1 mM PMSF). Peak N1R and N1RAsn26ΔAla containing protein fractions were stabilized by the addition of glycerol to a final concentration of 10% and Triton-X-100 was added to a 1% concentration to promote further solubilization and eliminate possible non-specific binding of the extract(s) to the dsDNA-cellulose columns. Extracts were incubated with Triton-X-100 for 20 mins on ice, prior to clarification of the extracts by a final centrifugation (4, 500 rpm, 5 mins; Beckman GS-15).

1 ml of the clarified extracts was loaded onto 1 ml packed native DNA cellulose (Pharmacia Biotech) prepared in buffer-Y (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 3 mM 2-mercaptoethanol, 1% Triton-X-100, 1 mM PMSF and 10% glycerol). Extracts were allowed to bind for 20 mins before being washed with 6 mls of buffer-Y at a flow rate of 2

ml/hour. Samples were then eluted stepwise with buffer-Y containing 0.1 M, 0.25 M, 0.5 M, 0.75 M, 1.0 M, and 1.5 M NaCl. All procedures were performed at 4°C. Fractions were analyzed for protein content using the Bradford (Bradford, 1976) Assay (Sigma Chemical) and for the presence of N1R or mutant N1RAsn26ΔAla by western blotting using mAb H1119 (1: 2000) after SDS-PAGE and electroblotting to nitrocellulose.

BIOINFORMATIC ANALYSIS

Alignments and structure modeling of the SFV N1R RING finger motif with HSV ICP0 were performed using the LOOK suite of programs (Molecular Applications group, Palo Alto, CA, USA) on a Silicon Graphics Indy Computer. Database searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990). Alignment of the amino acid sequences of N1R with ribbon-helix-helix family members was initially carried out using the Clustal W alignment program (Thompson *et al.*, 1994).

The consensus alignment of the N-terminal region of protein N1R with the cowpox and EV homologues and members of the MetJ family was then created manually by taking input from database search programs and multiple alignment programs. The positional percentage identity between sequences was calculated from the number of identical residues between aligned sequences; insertions and deletions were not used in these calculations. Two programs were used to predict the protein secondary structure of these polypeptides. Both use more than one method and perform the prediction on multiply aligned proteins to improve accuracy. Jnet (http://circinus.ebi.ac.uk:8081/jnet/) makes use of a neural network method and provides a secondary structure prediction using the multiple protein alignment and also a HMM profile (Cuff and Barton, 1999). Jpred (http://circinus.ebi.ac.uk:8081/index.html) works by combining a number of high quality

prediction methods (dsc, mulpred, nnssp, phd, zpred) to form a consensus (Cuff *et al.*, 1998).

RESULTS

Structural modeling of the poxvirus RING finger motif

NMR studies using two isolated RING finger motifs, one from the ICP0 protein of EHV (Everett *et al.*, 1993) and the other from the PML protein (Borden *et al.*, 1995), initially described slightly different structures for these motifs. However, data from molecular modeling indicates that the PML protein can be modeled on the EHV structure and that this is likely to be the correct fold for the RING finger (Bienstock *et al.*, 1996). Since the zinc coordination scheme of the ICP0 RING finger is distinct from other zinc binding proteins in that it uses alternate pairs of coordinating amino acids to bind two zinc ions in a cross brace, it was of interest to determine whether the poxvirus RING finger sequences were also compatible with this motif structure. Amino acids from the RING finger motifs of the poxvirus proteins (SFV, MYX, VV and EV) were aligned with those from ICP0, PML and several other proteins to generate an optimized alignment for modeling (Figure 1).

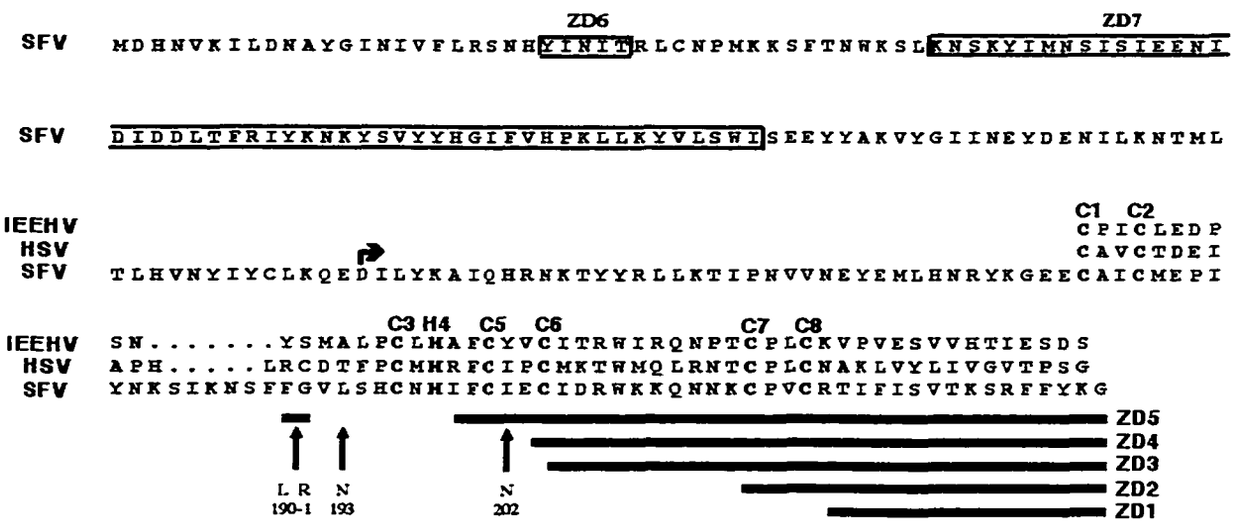


Figure 1. SFV N1R protein modifications. The complete amino acid sequence is shown for the SFV N1R protein. C-terminal deletions are indicated by the black bars (ZD1-5, respectively). Internal deletions (boxed) of amino acids #24-28 and #45-95, the end of the N-terminal deletion of 154 amino acids (right arrow) and positions of site specific mutations (vertical arrows) are indicated. Shown above the SFV sequence are the amino acid sequences

of 1) the RING motif from HSV-1 used to create the SFV-HSV fusion protein and 2) the RING motif from EHV.

The LOOK program was used to model the poxvirus sequences on the ICP0 structure. In each case the poxvirus sequence was successfully modeled with the preservation of the two zinc binding regions and the alpha helix. The amino acid insertions in the poxvirus proteins were modeled as loops away from the zinc binding regions and the alpha-helix. As an example, the predicted SFV RING finger is compared to the structure of the ICP0 RING finger in Figure 2.

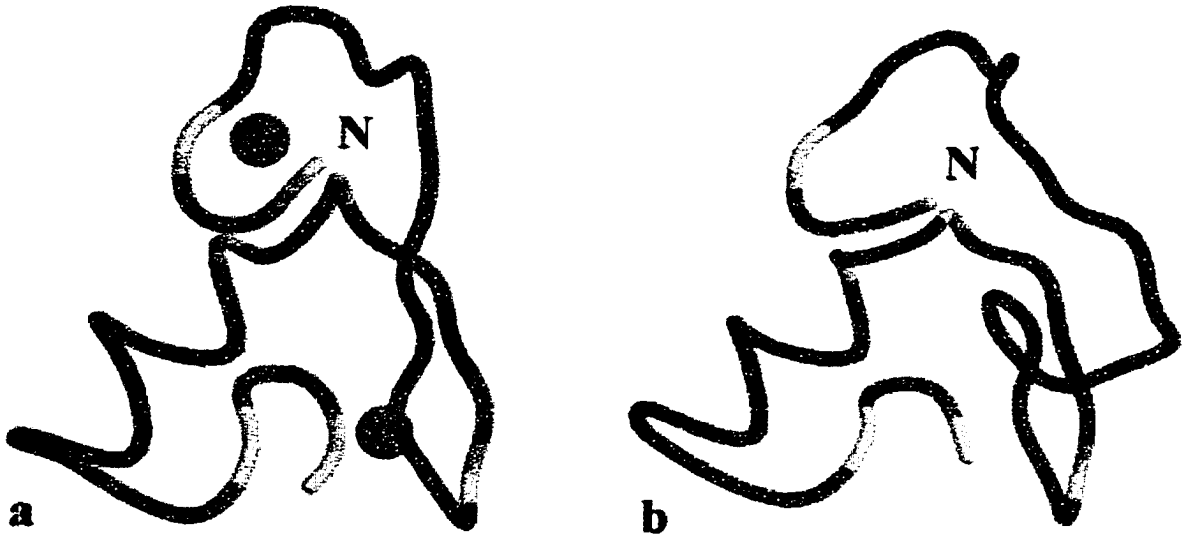


Figure 2. RasMol (Sayle and Milner-White, 1995) cartoons of molecular models showing backbone of RING domains. A) HSV-1 ICP0 RING domain. B) SFV N1R RING domain. Cysteines are in light gray; histidines are in black; zinc ions are represented as spheres; N indicates N-terminus

Although this data supports the hypothesis that the poxvirus RING fingers are similar to the cross-brace described for the ICP0 RING finger, it must be noted that this structure was determined using the isolated peptide. Indeed, the crystal structure of the

RAG1 (recombination-activating protein in lymphoid cells) dimerization domain suggests that the RING finger of this protein is not a discrete domain, but is a component of a larger protein structure (Bellon *et al.*, 1997).

Carboxy-terminal deletion analysis of the SFV N1R RING motif.

To determine the role of the RING motif in the localization of N1R to virus factories, modified N1R proteins with C-terminal truncations (Figure 1) were transiently expressed in VV infected cells. The vectors were based on pMSN1 (Upton *et al.*, 1994) that uses the strong late promoter of pMJ601 (Davison and Moss, 1990). Localization of the modified proteins was determined by immunofluorescence microscopy.

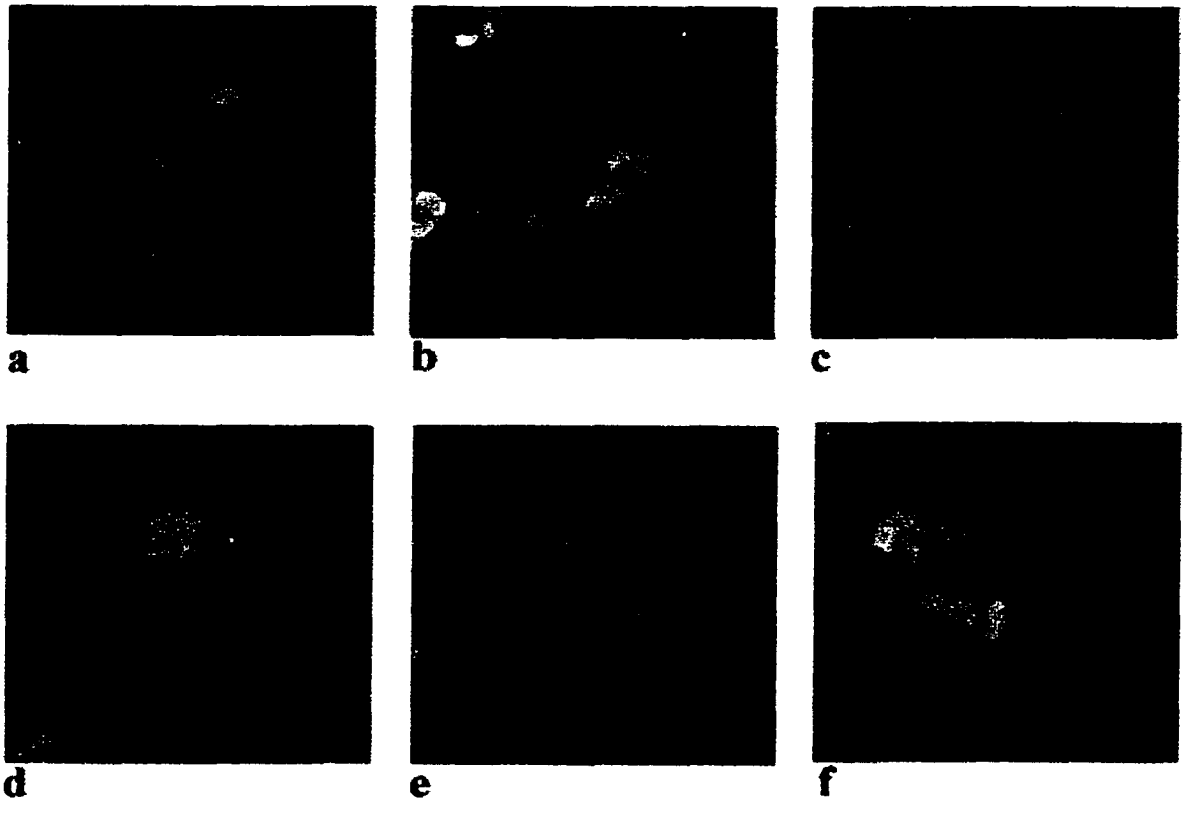


Figure 3. Localization of SFV N1R protein in VV infected cells shown by mAb H1119 and confocal microscopy. The protein expressed from the transfected vectors are as follows: a) wild type SFV N1R; b) N1R-ZD4; c) N1R-ZD5; d) N1R-HSV fusion; e) N1R-d24-28; f) N1R-mL193N (m; site specific mutant).

Deletions ZD1, ZD2, ZD3 and ZD4 which remove progressively more of the C-terminal portion of N1R up to and including the third distal cysteine of the RING motif, had little or no effect on the localization of N1R to the factories (Figure 3; ZD1, ZD2 and ZD3 not shown). Deletions ZD1 and ZD2 effect only the last pair of coordinating cysteines, but the third distal cysteine is predicted to be involved in coordinating the first zinc atom with the first cysteine pair. The larger deletion, ZD5, which includes the fourth distal cysteine abolished localization to the virus factory (Figure 3c). Thus the small difference between deletions ZD4 and ZD5 delineates a region of the protein that is required to permit normal localization to virus factories. Each of the mutants created here and others in following experiments were shown to be expressed at levels comparable to normal N1R and to be the correct size by western blots of extracts of transfected VV infected cells (Figure 4).

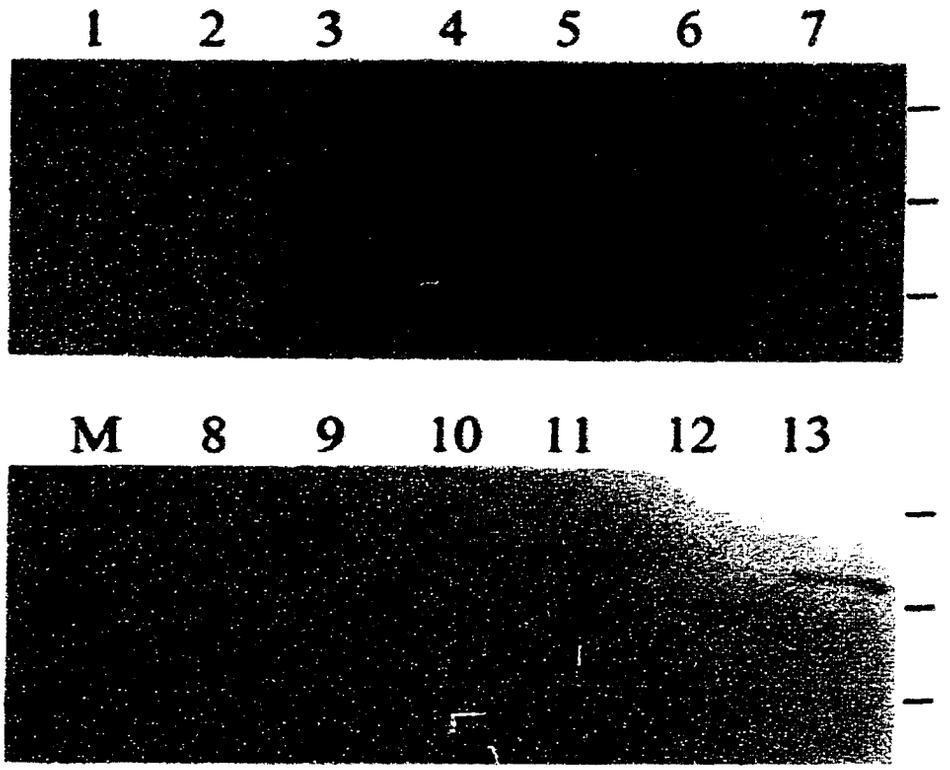


Figure 4. Western blot analysis of transient expression of SFV N1R mutant proteins in VV infected BGMK cells. Lanes: 1) SFV-N1R (wild type); 2) N1R-ZD1; 3) N1R-ZD2; 4) N1R-ZD3; 5) N1R-ZD4; 6) N1R-ZD5; 7) N1R-ZD6; 8) N1R-mFG190/191R; 9) N1R-mL193N; 10) N1R-mI202N; 11) N1R-ZD6; 12) N1R-ZD7; 13) N1R-HSV; M) Standard proteins (and bars), 32.5, 25, 16.5 kDa.

In an attempt to correlate localization to virus factories with zinc binding, a zinc blot of *E. coli* expressed N1R and deletion proteins was performed (Figure 5).

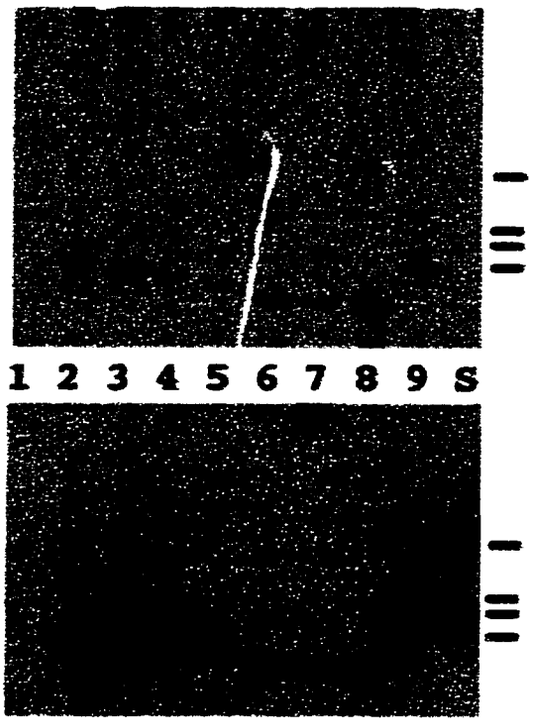


Figure 5. Analysis of protein expression and zinc binding by SFV N1R proteins with C-terminal deletions. Top panel, Coomassie blue stained polyacrylamide gel of SFV N1R proteins isolated as inclusion bodies expressed in *E. coli*. Bars indicate standards (top to bottom: 40 kDa - alcohol dehydrogenase (positive control for zinc binding), 35 kDa - carboxypeptidase A (negative control for zinc binding), 29 kDa - carbonic anhydrase (positive control for zinc binding) and SFV N1R protein. Bottom panel, autoradiogram of a zinc blot from a gel identical to that shown in the top panel. Lanes: 1) bacterial vector pET19b; 2) MYX N1R ortholog; 3) SFV N1R; 4) SFV N1R-ZD1; 5) SFV N1R-ZD2; 6) SFV N1R-ZD3; 7) SFV N1R-ZD4; 8) SFV N1R-ZD5; 9) SFV N1R; 10) Standard proteins.

All of the proteins, including deletion ZD5 that failed to localize to the virus factory, bound zinc in this assay. Since deletion ZD5 removes both the third and fourth pairs of cysteines, binding of zinc at both sites should be blocked if the SFV N1R is folded in a cross-brace structure (Borden *et al.*, 1995; Borden and Freemont, 1996; Everett *et al.*,

1993; Saurin *et al.*, 1996). Although this result suggests that the cross-brace is not used by SFV N1R, the zinc blot assay relies on the renaturation of protein after blotting from a SDS polyacrylamide gel, and it is therefore possible that the observed zinc binding results from an aberrant folding of the remaining portion of the RING motif region which still contains three cysteines and a histidine. Molecular modeling indicates a cross-brace structure for the poxvirus RING motif is possible, but determination of its structure by NMR or crystallography may be required to resolve this issue.

Effect of site-specific mutations on localization of the SFV N1R protein.

Since C-terminal deletions up to the third distal cysteine did not effect localization we made two single amino acid changes and one two amino acid change at non-cysteine positions conserved between the SFV and VV sequences in the central part of the SFV N1R RING motif. Plasmids N1R-mL193N, N1R-mI202N and N1R-mFG190/191LR replace the following amino acids in the SFV N1R protein, leucine-193 with asparagine, isoleucine-202 with asparagine and phenylalanine-190 and glycine-191 with leucine and arginine respectively. Following transfection into VV infected cells, these mutant proteins localized to virus factories (Figure 3f), indicating that although these residues are conserved between SFV and VV, these substitutions are compatible with localization. These results prompted us to question whether other regions of the N1R protein outside of the RING motif were directly involved in localization to virus factories, and whether the N1R RING finger motif could be replaced by another from a non-poxvirus protein.

Deletion of the N-terminal 154 amino acids of SFV N1R (slightly more than half of the protein; Figure 1) blocked localization demonstrating that the RING motif region is not sufficient for association with the virus factory (data not shown). Similarly, deletion of amino acids #45-95 from SFV N1R (Figure 1) resulted in loss of virus factory localization (data not shown). In an attempt to delineate a smaller region of the N-terminal region of

SFV N1R that might be involved with localization we examined an alignment of the poxviral N1R proteins (Figure 6).

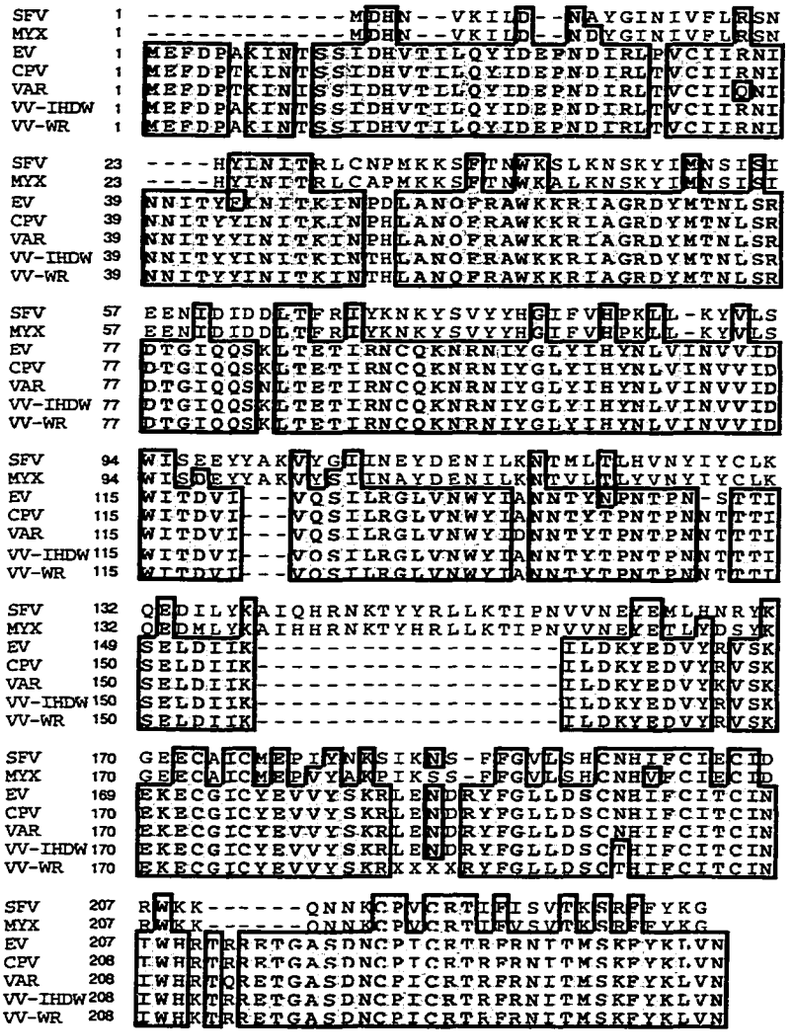


Figure 6. Alignment of SFV N1R protein sequence with poxviral orthologs. Boldfaced letters indicate the highly conserved block of N-terminal residues and the coordinating Cys and His residue(s) of the RING finger motif. Abbreviations: SFV, Shope fibroma virus, accession number L26342; MYX, Myxoma virus, accession number AF170726; EV, Ectromelia virus p28 accession number U01161; CPV, Cowpox virus accession number Y11842; VAR, Variola major virus accession number L22579; VV, Vaccinia virus (strain WR, western reserve)

accession number M22812. The position of the premature stop codon introduced by a frameshift mutation in the VV-WR gene sequence is indicated by XXXX.

There is only 28% amino acid identity between SFV and VV N1R protein orthologs and the region with the greatest similarity is at the C-terminus, in the region of the RING finger motif. There is, however, one block of five absolutely conserved amino acids close to the N-terminus of SFV N1R (residues #24-28; Figure 1). This sequence, Tyr-Ile-Asn-Ile-Thr, was deleted from SFV N1R by site-specific mutagenesis in plasmid N1R-d6. The deletion of this small region prevented the SFV N1R protein from localizing to the virus factory in VV transfected cells (Figure 3e). Thus, factory localization requires both a small region at the N-terminus of the protein and part of the RING finger motif.

Although the RING motif has been observed in a great variety of proteins, its function has remained largely unknown. In order to test the hypothesis that these motif regions may fulfill similar functions in proteins containing this motif, a chimeric gene was constructed to express a protein that had the N1R RING motif replaced by the RING motif of herpes simplex virus type-1 (HSV-1) immediate early protein ICP0. Transfection of this construct (N1R-HSV) into VV infected cells demonstrated that this chimeric protein did indeed localize to discrete regions within infected cells (Figure 3d).

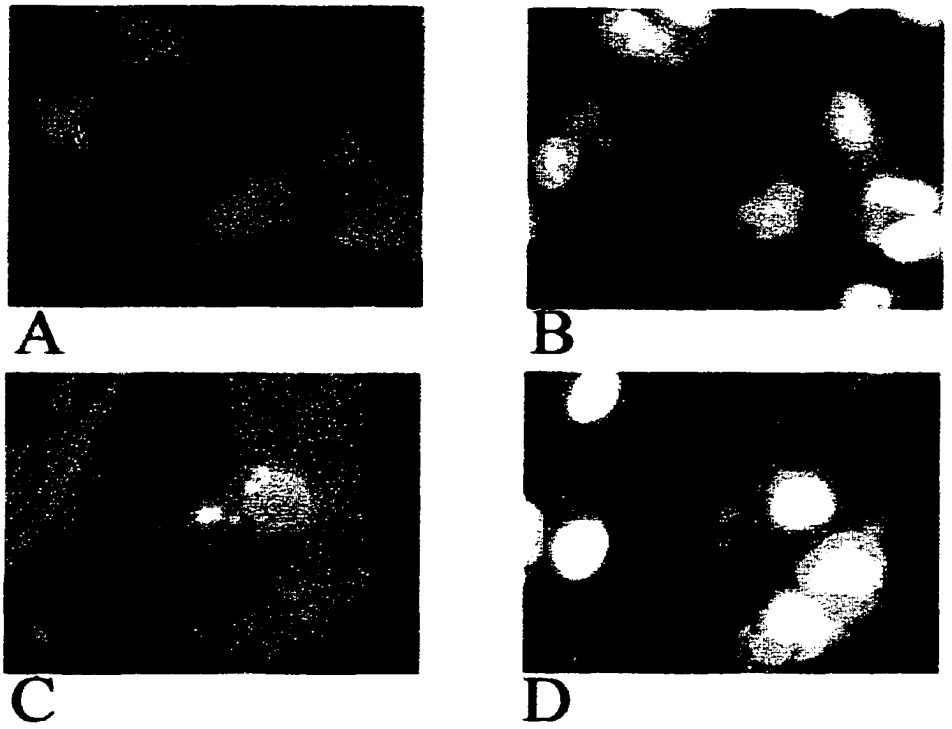


Figure 7. The HSV-1 ICP0 RING motif can replace the SFV N1R RING motif for virosome localization. Vectors (SFV N1R panels A and B; N1R-HSV panels C and D) were transfected into VV infected cells and localization determined by immunofluorescence. Panels A and C, protein detection by immunofluorescence; panels B and D, identical fields visualized for DNA stained with bisBenzimide. Virus factories are indicated by asterisks.

Since ICP0 is a nuclear protein, it was confirmed that the site of localization was the virus factories outside the nucleus by using Hoechst dye 33342 to stain both the viral DNA and the host nuclear DNA (Figure 7). Thus, the HSV ICP0 RING motif can replace the SFV N1R RING motif in supporting factory localization.

SFV N1R protein binds to both ds- and ss-DNA cellulose

In order to test whether the SFV N1R protein is localized to the virus factory because of binding to viral DNA or an affinity for another virus factory protein, a recombinant VV overexpressing SFV-N1R was constructed. Immunoprecipitation analysis

using mAb H1119 was carried out on VV-N1R and VV (as negative control) infected BGMK cell lysates using a variety of methods (Figure 8).

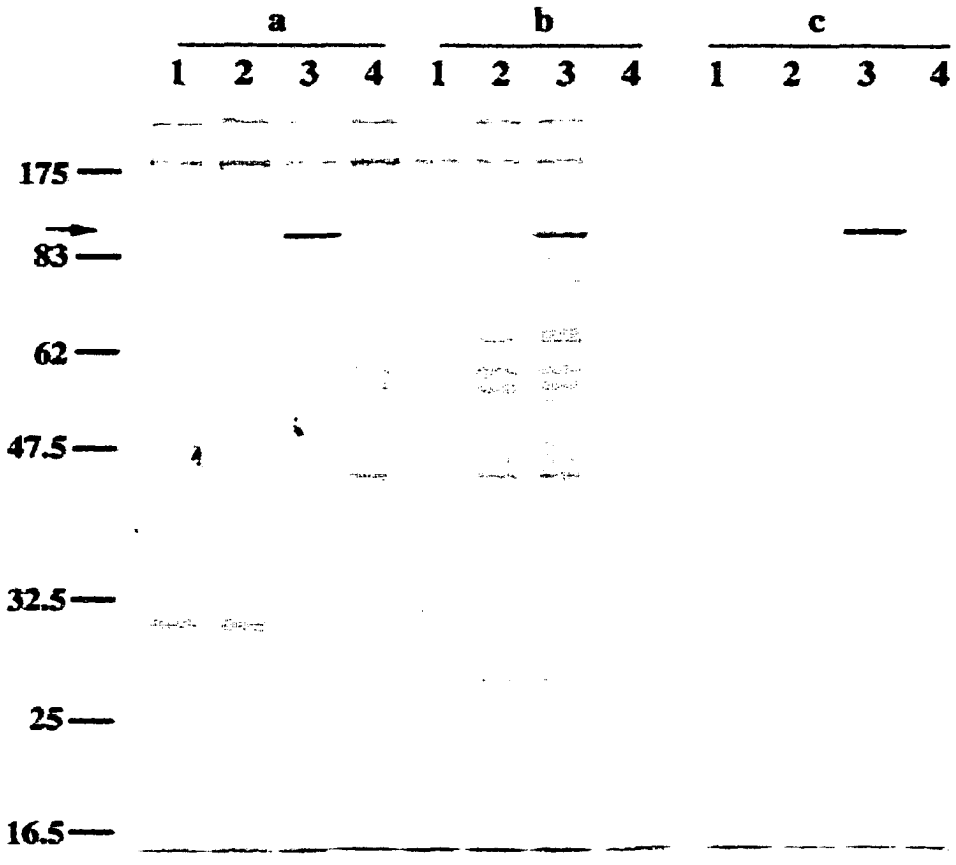


Figure 8. Autoradiograph of immunoprecipitation analysis of VV infected BGMK cell lysates using mAb H1119. ^{35}S-labelled VV or VV-N1R infected cell lysates were immunoprecipitated according to methods a (Ciccone *et al.*, 1988), b (Rothe *et al.*, 1994) and c (de Gunzburg *et al.*, 1989). Lanes: 1) VV-N1R infected (mAb H119); 2) VV infected (mAb H1119); 3) VV-N1R infected (mAb 5013); 4) VV infected (mAb 5013). Arrow indicates the position of beta-galactosidase

N1R failed to immunoprecipitate from VV-N1R infected BGMK cells using mAb H1119 (which can immunoprecipitate HSV-1 ICP27; S. Rice, personal communication), although beta-galactosidase (Figure 8a, 8b, 8c; lane 3), which is also expressed by VV-

N1R, immunoprecipitated under the conditions tested. Although it is possible that the SFV N1R N-terminal epitope tag is buried within the native structure of N1R and as such inaccessible to mAb H1119 under non-denaturing conditions, it is more likely that the failure to immunoprecipitate N1R resulted from a strong DNA binding activity of N1R (see below).

For DNA cellulose chromatography procedures, recombinant virus VV-N1R was used to produce the SFV N1R protein, all of which localized to virus factories when examined by immunofluorescence (data not shown). Little soluble N1R protein was observed in lysates of infected cells; N1R remained tightly associated with the rapidly sedimenting virosome pellet fraction. Initial attempts to release N1R from the pellet fraction involved the addition of increasing concentrations of urea or NaCl to the lysate. Urea concentrations up to 8 M failed to release N1R (data not shown), but the protein was extracted when 0.6 M NaCl was included in the buffer (Figure 9).

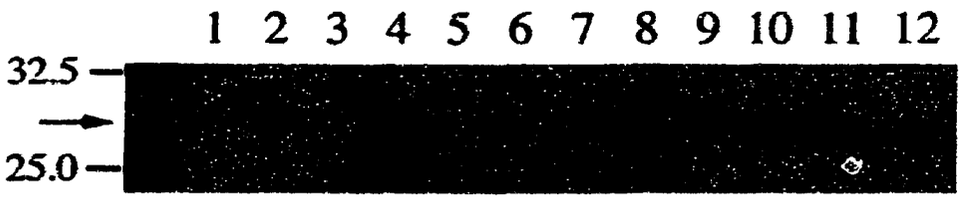


Figure 9. Western blot showing extraction of N1R from the virosome pellet using NaCl. VV-N1R infected BGMK cells were harvested, and lysates were incubated with increasing NaCl concentration prior to centrifugation (14,000 g, 5 mins). Pellet (Lanes 1-6) and cytoplasmic (Lanes 7-12) fractions were separated by SDS-PAGE and the presence of N1R detected by Western blotting using the mouse mAb H1119. Buffer was NETP (1 mM EDTA, 50 mM Tris-HCl [pH 8.0], 3 mM beta-mercaptoethanol). Lanes: 1 and 7) No NaCl; 2 and 8) 0.2 M NaCl; 3 and 9) 0.4 M NaCl; 4 and 10) 0.6 M NaCl; Lanes 5 and 11) 0.8 M NaCl and Lanes 6 and 12) 1.0 M NaCl. Standard molecular weight proteins are indicated with bars (kDa).

Initial attempts at preparing extracts for DNA cellulose columns were carried out according to a classical method (Alberts and Herrick, 1971), however, the addition of PEG 6000 to precipitate cellular DNA was found to destroy the ability to detect epitope tagged N1R by western blot using mAb H119 (data not shown). An alternative strategy was then employed to shear DNA using a fine gauge needle and eliminate salt from the extract using desalting columns.

Desalted protein extracts were loaded onto ds- and ss-DNA columns and eluted with NaCl step gradients. A western blot showing SFV N1R protein binding to and eluting from ds-DNA (between 0.5 - 0.75 M NaCl) is shown in Figure 10a. A similar profile was seen for ss-DNA (Figure 10b).

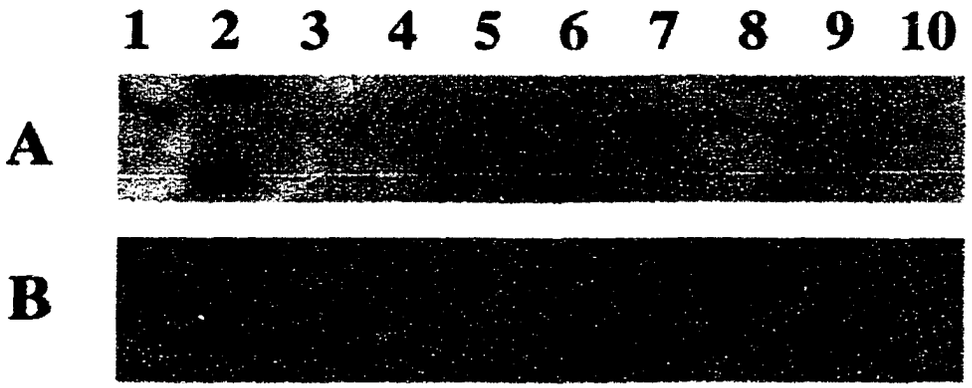


Figure 10. Western blot showing binding of SFV N1R protein to ds- (A) and ss-DNA (B) cellulose. Buffer was 50 mM NaCl, 3 mM 2-mercaptoethanol, 10 mM $ZnCl_2$, 1 mM PMSF, 50 mM Tris (pH 8.0). Lanes: 1) protein extract loaded on to column; 2) MW standards 32.5 kDa and 25.0 kDa; 3) recycled flow through; 4) wash, no NaCl; 5) 0.1 M NaCl; 6) 0.25 M NaCl; 7) 0.5 M NaCl; 8) 0.75 M NaCl; 9) 1.0 M NaCl; 10) 1.5 M NaCl.

When 1 mM EDTA was included in the desalting buffer the protein did not bind to ds- (Figure 11A) or ss-DNA (Figure 11B) columns suggesting that zinc and the structural integrity of the RING finger motif region are required for N1R's DNA binding activity.

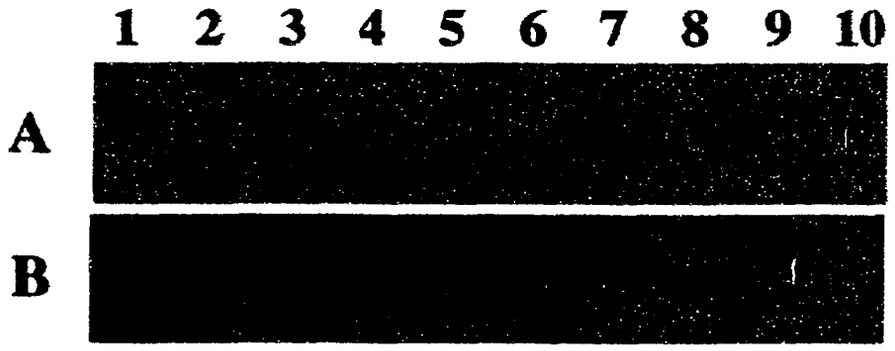


Figure 11. Western blot showing inhibition of binding of SFV N1R protein to ds- (A) and ssDNA (B) cellulose by EDTA. Buffer was 50 mM NaCl, 1 mM EDTA, 3 mM 2-mercaptoethanol, 10 mM $ZnCl_2$, 1 mM PMSF, 50 mM Tris (pH 8.0). Lanes: 1) protein extract loaded on to column; 2) MW standards 32.5 kDa and 25.0 kDa; 3) recycled flow through; 4) wash, no NaCl; 5) 0.1 M NaCl; 6) 0.25 M NaCl; 7) 0.5 M NaCl; 8) 0.75 M NaCl; 9) 1.0 M NaCl; 10) 1.5 M NaCl.

Although it is possible that SFV N1R protein localizes to the virosome and binds DNA via an associated viral protein, this appears unlikely since the poxviral N1R protein orthologs, which are poorly conserved overall, all localize in cells infected by distantly related poxviruses.

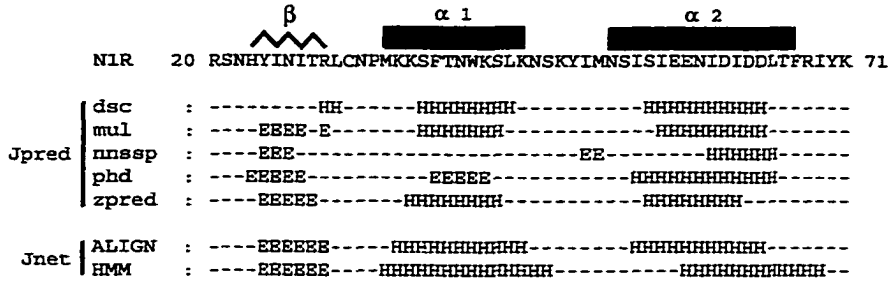
N1R contains a Ribbon-Helix-Helix Motif

The observation that a chimeric protein, in which the RING finger of the herpes simplex virus-1 ICP0 protein replaced the RING finger of SFV N1R also localized to the virus factories, indicated that regions outside of the RING finger of N1R likely governed the specificity of factory localization since the herpes virus ICPO protein normally associates with the nucleus. Outside of the RING finger region of the poxviral proteins, the conservation of the N-terminal block of five amino acids (residues 24-28 of the SFV-N1R protein) indicated this region was likely to be of functional importance in the context of viral infection. Preliminary structural prediction analysis indicated this five amino acid sequence of N1R likely formed a short beta-strand region. Inspection of the literature identified a small family of DNA binding proteins, termed the ribbon-helix-helix family, that utilize a short beta-sheet for specific interaction with DNA (Figure 12).

Figure 12. RasMol (Sayle and Milner-White, 1995) cartoon of Salmonella phage P22 Arc protein beta-sheet DNA interaction. Operator site DNA is represented by the white double helix; β sheet regions are represented in light gray and helices in dark gray. The left side of the diagram shows a space filled model of β sheet-DNA interaction; the right side shows the basic backbone structure.

Alignment of N1R with members of this family revealed considerable similarity (Figure 13B).

A.



B.

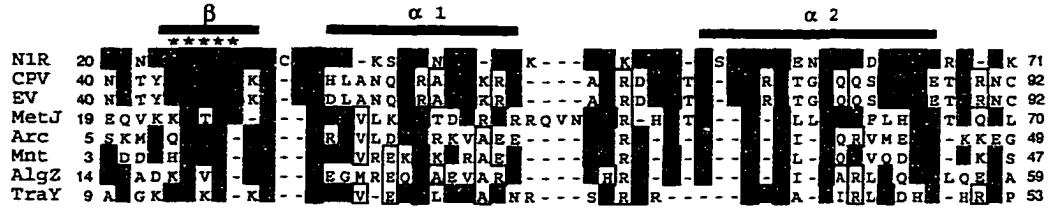


Figure 13. Structural prediction and sequence alignments of the ribbon-helix-helix family. A, Prediction of protein secondary structure by Jpred and Jnet using a multiple alignment of N1R, poxviral orthologs and ribbon-helix-helix family members. Locations of secondary structure observed in the known structure of Arc are located above the aligned partial amino sequence of N1R. Amino acid regions are predicted either to form either a consensus β-strand (denoted by letter E) or a helical (denoted by letter H) regions. B, alignment of N1R and poxviral orthologs with ribbon-helix-helix proteins. The partial amino acid sequences of Met J (residues 19-70), Arc (residues 5-49), Mnt (residues 3-47), AlgZ (residues 14-59) and TraY (residues 9-53) are aligned with the N-termini of N1R (residues 20-71), and orthologous proteins from ectromelia virus (residues 40-92) and cowpox virus (residues 40-92). Alignments were performed manually by taking input from several database search and

multiple alignment programs. Locations of secondary structural elements are indicated using bars above the sequence alignment. Asterisks above the β strand region indicate those N1R amino acids (Tyr-24, Ile-25, Asn-26, Ile-27 and Thr-28) that were individually mutated to Ala.

In all cases, the arrangement of hydrophobic buried residues alternating with polar exposed residues of the beta-strand is conserved, consistent with the nature of β-sheet DNA interaction where alternating residues face the DNA (Suzuki, 1995). Additionally, the Ser residue at the amino terminus of helix-B, which was observed in the crystal structure of the MetJ repressor-operator complex (Somers and Phillips, 1992) to make important docking side chain hydrogen bonds with the phosphate backbone is absolutely conserved between N1R, its poxviral orthologs and members of this DNA-binding family. The overall sequence identity between Arc, Mnt, and MetJ is low, with only five residues totally conserved. This is most likely because the proteins have evolved to recognize different DNA sequences (Raumann *et al.*, 1994); however, structural studies have shown that these proteins form homologous three dimensional ribbon-helix-helix folds.

The consensus from structural prediction programs strongly suggests that the N-terminal region of N1R and the poxviral orthologs can adopt a similar fold to members of this ribbon-helix-helix protein family (Figure 13A). Similarly, a quantitative analysis of the alignment presented in Figure 13B provided further evidence for assigning N1R to this protein family (Table 2).

	N1R	CPV	EV	MetJ	Arc	Mnt	AlgZ	TraY
N1R		17	16	8	8	11	9	6
CPV	35		51	8	7	8	8	7
EV	32	96		8	7	8	8	7
MetJ	18	17	17		9	11	7	9
Arc	19	16	16	21		19	13	10
Mnt	26	18	18	25	42		14	12
AlgZ	21	17	17	16	29	31		12
TraY	14	16	16	21	24	29	29	

Table 2. Numerical analysis of alignments between N1R, poxviral orthologs and members of the ribbon-helix-helix family of DNA-binding proteins. Upper entries represent the number of identical residues between aligned pairs. Lower entries represent the percentage identity between alignments.

The most similar members of this family are Arc, Mnt and AlgZ. N1R is most similar to Mnt, AlgZ, Arc and MetJ, whereas the fit to TraY is low. Interestingly, over the aligned region, N1R exhibits a degree of similarity to Mnt that is comparable to the similarity between Mnt and the structurally homologous MetJ repressor providing reinforcement that this N-terminal region of N1R may indeed take on a similar structure. If N1R is indeed a member of the ribbon-helix-helix family of proteins, then alteration of β-strand residues should directly effect the localization of N1R to the DNA containing viral factories. Indeed, as previously presented, deletion of this region from N1R resulted in a complete lack of factory localization (Figure 3e) making it an ideal candidate region for site-directed mutagenesis studies. Site-specific pMSN1 mutants of the SFV N1R β-strand region were constructed and their localization pattern following transient expression in VV infected cells determined by indirect immunofluorescence (Figure 14).

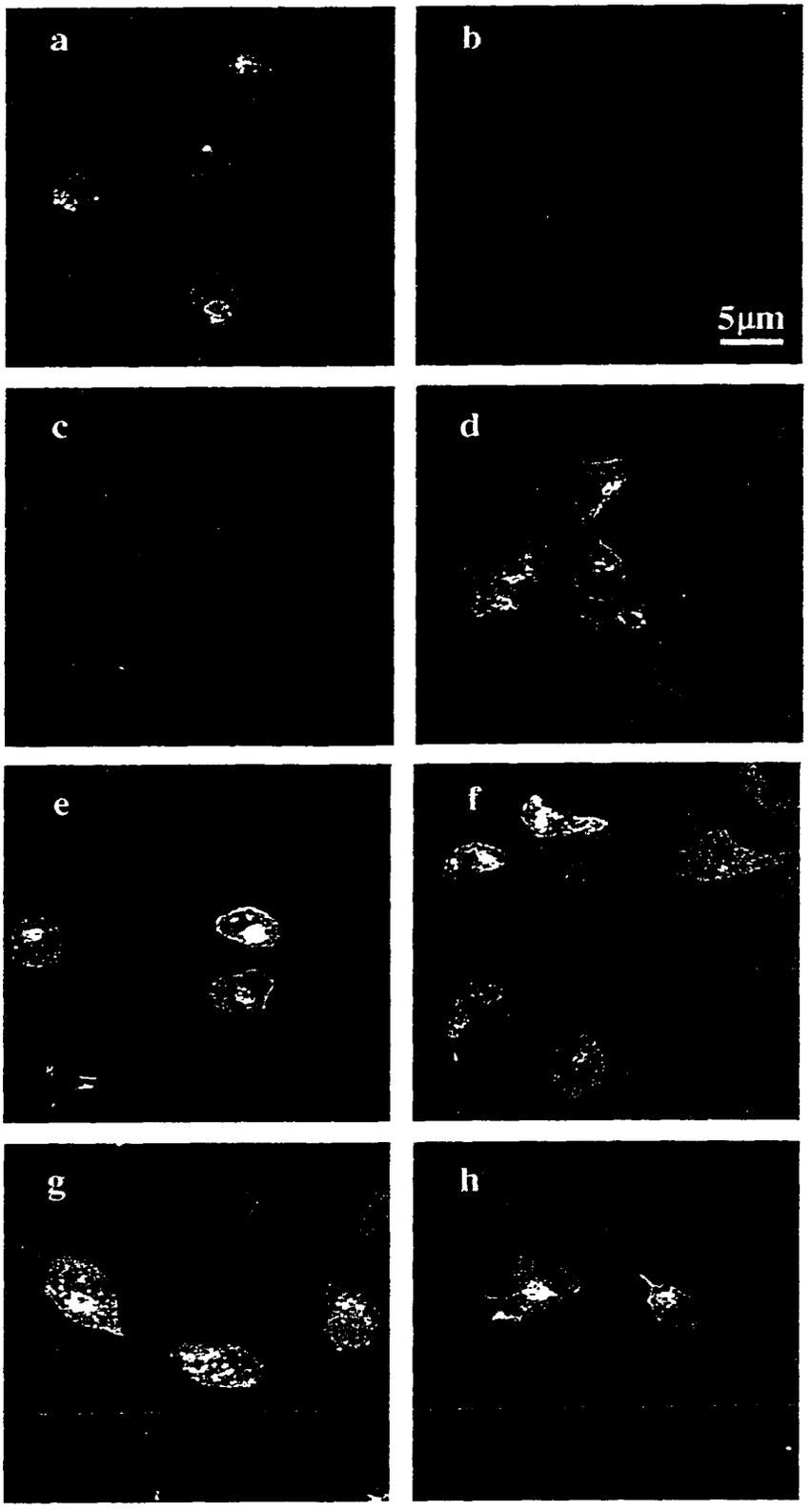

a
b
5μm
c
d
e
f
g
h

Figure 14. Localization of SFV N1R site-specific alanine mutant proteins in VV infected cells shown by mAb H1119 and confocal microscopy. The protein expressed from the transfected vectors are as follows: a) wild type SFV N1R; b) no transfection; uninfected cells; c) no transfection; VV infected cells; d) N1R-Tyr24ΔAla; e) N1R-Ile25ΔAla; f) N1R-Asn26ΔAla; g) N1R-Ile27ΔAla; h) N1R-Thr28ΔAla.

Wild type N1R localized to defined foci (the virus factories) within the cytoplasm of VV infected cells (Figure 14a), but mutation of any of the 24-Tyr-Ile-Asn-Ile-Thr-28 amino acids individually to Ala, significantly reduced localization (Figure 14d-h). Substitution of Tyr-24, Ile-25 and Thr-28 had relatively modest disruptive effects on localization, whereas mutation of Asn-26 consistently had the greatest effect with little or no virus factory localization seen. The N1R-Asn26ΔAla mutant protein was observed throughout the cytoplasm (Figure 14f). Interestingly, the distal Ile-27 residue also has a large effect on localization (Figure 14g). In order to confirm that the observed degree of localization of the mutant proteins to the factories was not due to alteration of protein stability or increased protease sensitivity, expression of the altered proteins was characterized. Each of the mutant proteins was expressed at levels comparable to the wildtype SFV-N1R and to be the correct size using western blots of extracts of the transfected VV infected cells (Figure 15).

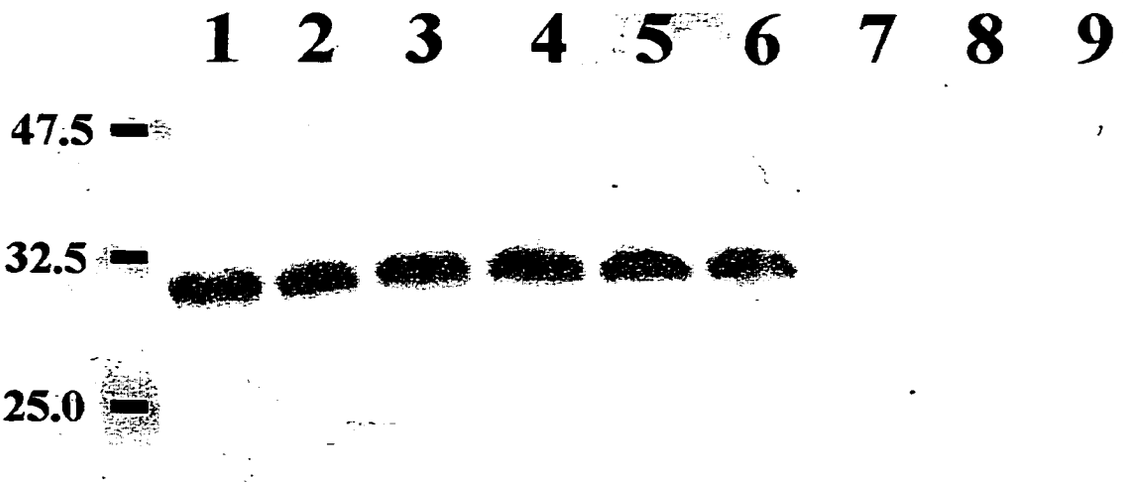


Figure 15. Detection of N1R and Ala specific N1R mutant expression by western blot analysis. VV infected BGMK cells were transfected with vectors and harvested after 24 hours. Proteins were detected with mAb H1119. Lanes: 1) SFV-N1R (wild type); 2) N1R-Tyr24ΔAla; 3) N1R-Ile25ΔAla; 4); N1R-Asn26ΔAla; 5) N1R-Ile27ΔAla; 6) N1R-Thr28ΔAla; 7) Uninfected; 8) Uninfected/transfected with SFV-N1R; 9) VV infected/no transfection. Standard proteins (in kDa) are denoted with bars.

Asparagine-26 is Important for the DNA-Binding Activity of N1R

The localization pattern of the SFV-N1RAsn26ΔAla mutant protein, throughout the cytoplasm, suggested that the predicted β-strand region of N1R was involved in DNA binding and thus factory localization. To test this hypothesis, a recombinant VV expressing the mutant SFV N1RAsn26ΔAla protein was constructed and following infection, the mutant protein was partially purified from infected cell lysates and tested for its ability to interact with ds DNA cellulose. In contrast to wild type N1R protein which was tightly associated with the rapidly sedimenting virosome fraction, the mutant N1RAsn26ΔAla protein remained in the supernatant fraction consistent with the failure of this mutant protein to localize to the virus factories. Mutation of SFV-N1R Asn26 to Ala also disrupted binding of N1R to DNA cellulose (Figure 16B).

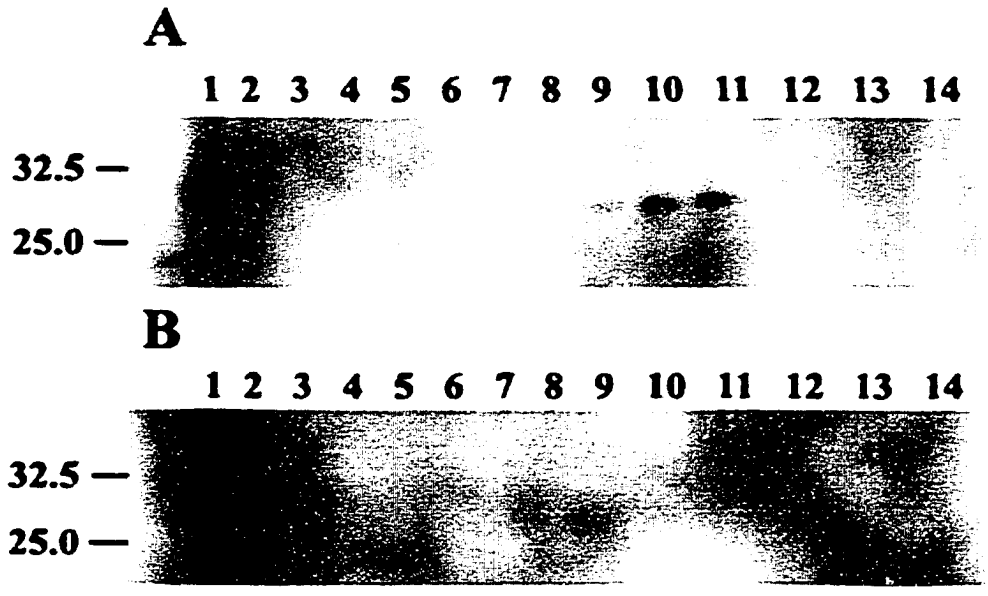
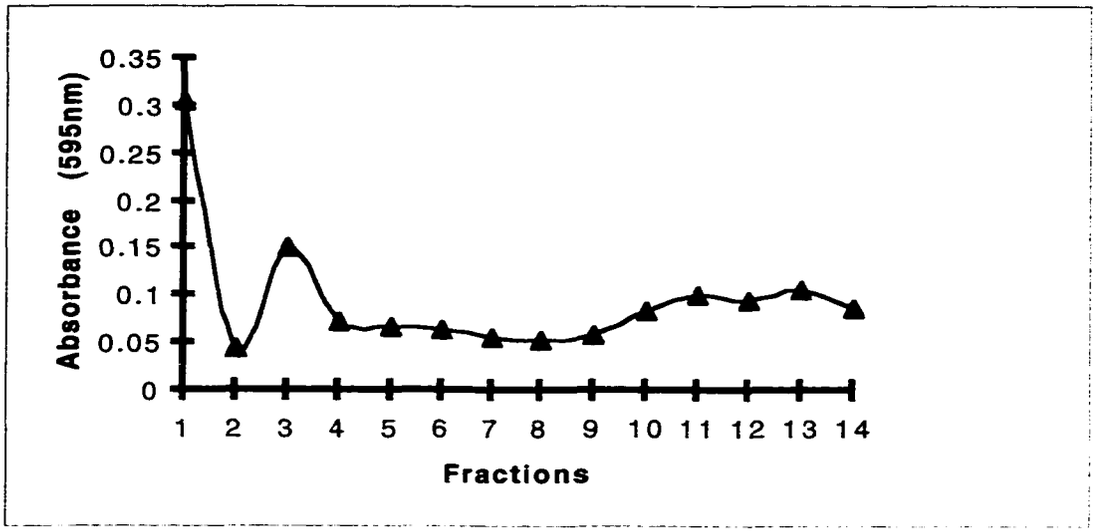


Figure 16. Western blot analysis of the interaction of N1R and mutant N1R-AsnΔAla protein containing VV infected BGMK cell extracts with ds-DNA-cellulose. VV-N1R or VV-N1RAsn26ΔAla virus infected BGMK cells were harvested, and soluble clarified, fractionated lysates containing N1R wildtype or the N1RAsn26ΔAla mutant protein were subjected to DNA-cellulose chromatography. Fractions were separated on SDS-PAGE and the presence of N1R (A) or mutant N1RAsn26ΔAla protein (B) was detected by Western blotting using the mouse mAb H1119 and enhanced chemiluminescence. Buffer was 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 3 mM 2-mercaptoethanol, 1% Triton-X-100, 1 mM PMSF and 10% glycerol. (Lanes) 1, Protein extract loaded on to column; 2-7, washes, no NaCl; 8, 0.1 M NaCl; 9, 0.25 M NaCl; 10, 0.5 M NaCl; 11, 0.75 M NaCl; 12, 1.0 M NaCl; 13, 1.5 M NaCl; 14, column void volume. Standard molecular weight proteins are indicated with bars (kDa).

The mutant protein was found in the first column washes (Figure 16B Lane 2) whereas SFV-N1R bound tightly to the DNA column, was undetectable in the wash fractions and was only eluted with 0.5-0.75M NaCl (Figure 16A. Lanes 10 and 11). Analysis of protein concentration in the fractions indicated that in both cases the majority of

the applied protein eluted from the columns with the first washes (Figure 17). This was further confirmed by SDS-PAGE of the individual fractions and Coomassie blue staining (data not shown). Thus, Asn26 within the predicted β-strand region of N1R is important for the DNA binding activity of N1R. The substitution of Ala for Asn effectively removes the hydrogen bonding potential of Asn through the elimination of the polar side chain. The specificity of the Asn-26 residue of N1R in DNA binding and localization is further substantiated by the nature of the Ala residue which is sufficiently short enough such that interference with other neighboring residues is unlikely. Thus, while a structured RING finger motif seems important for DNA binding and factory localization, the presence of the conserved poxviral amino terminal β strand region, together with site-specific mutational analysis of this region presented here, indicates this poxviral ribbon-helix-helix fold may be directly involved in DNA recognition.

A



B

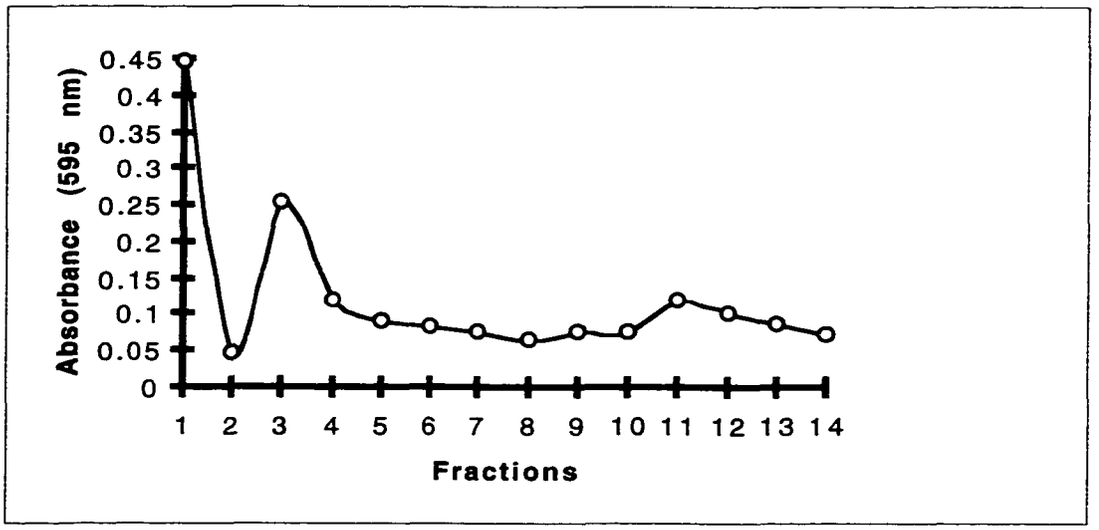


Figure 17. Protein elution profile of N1R and mutant N1R-AsnΔAla containing VV infected BGMK cell extracts from ds-DNA cellulose. A, SFV N1R containing extract; B, SFV-N1RAsn26ΔAla containing extract; 1.0 OD = 0.88 mg/ml protein. Fractions: 1) extract applied; 2) void volume 3) wash 1, no NaCl; 4) wash 2, no NaCl; 5) wash 3, no NaCl; 6) wash 4, no NaCl; 7) wash 5, no NaCl; 8) wash 6, no NaCl; 9) 0.1 M NaCl; 10) 0.25 M NaCl; 11) 0.5 M NaCl; 12) 0.75 M NaCl; 13) 1.0 M NaCl; 14) 1.5 M NaCl.

MYX N1R is transcribed both at early and late times post infection

In order to characterize the expression pattern of poxviral RING finger proteins following infection and to acquire useful biological tools to study their function, *E. coli* expressed N1R protein was used to raise monoclonal antibodies. 18 monoclonal hybridomas were obtained and utilized in western blots to screen for recognition of *E. coli* expressed N1R. Hybridoma supernatants #1A4, #2A1, #5A1, #7A8, #10C8 and #7D4 were found to recognize the *E. coli* expressed N1R protein in western blots and were subsequently screened by immunofluorescence for immunoreactivity to SFV infected or VV transfected BGMK cells. Hybridoma supernatants #5A1 and #7D4 showed reactivity towards the virus factories and as such these hybridomas were utilized to raise ascites fluid. However, in subsequent western blot analysis, while detection of *E. coli* expressed N1R protein occurred, no specific cross reactive signal was detected from SFV infected BGMK cell lysates (Figure 18 Lanes 5-10).

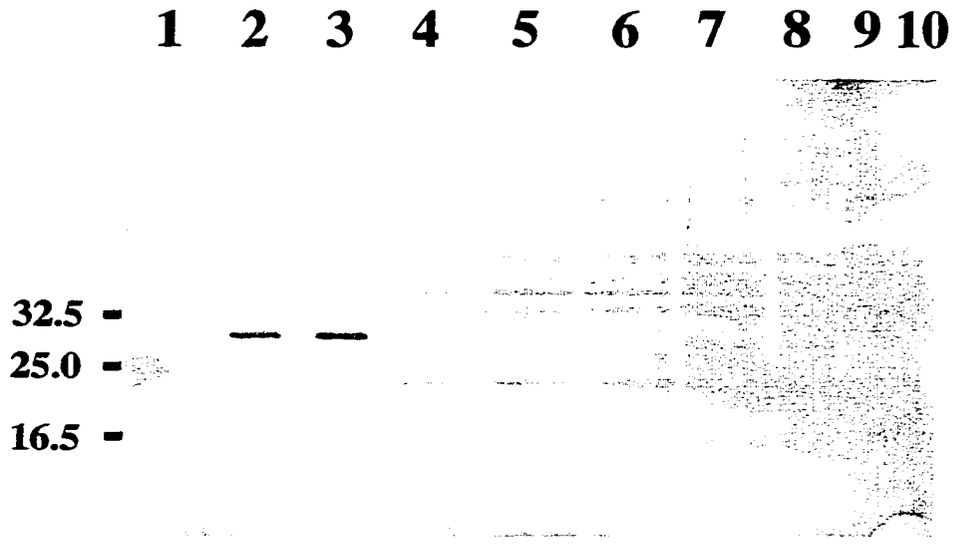


Figure 18. Western blot analysis of SFV infected BGMK cell lysates using mAb # 7D4 ascites fluid. Lanes: 1) pET19b (- control); 2) pET19b MYX-N1R (+ control); 3) pET19b SFV-N1R (+ control); 4) uninfected BGMK; 5) SFV infection (1 hour); 6) SFV infection (2 hour); 7) SFV infection (3 hour); 8) SFV infection (4 hour); 9) SFV infection (5 hour); 10) SFV infection (6 hour).

Northern blot analysis (Figure 19), however, indicated the MYX N1R gene is transcribed as soon as one hour post infection and at later times as a higher molecular weight transcript, consistent with transcriptional readthrough by viral encoded RNA polymerase (Moss, 1996b). This result concurs with EVp28 expression studies, which indicated EVp28 is expressed throughout the infection cycle (Senkevich *et al.*, 1995).

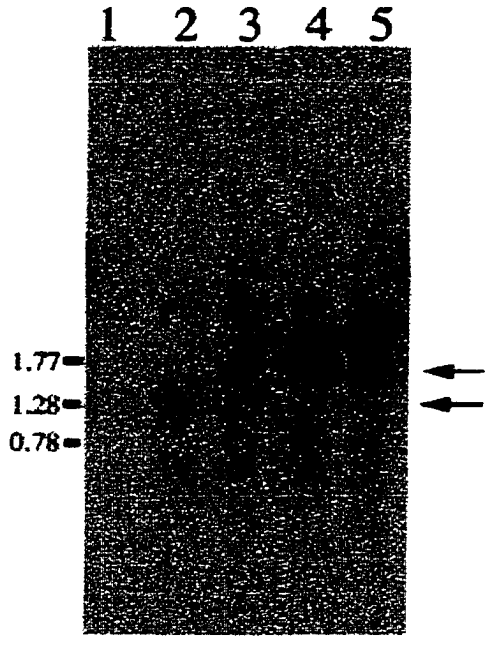


Figure 19. Northern blot analysis of MYX N1R mRNA expression following infection of BGMK cells. Northern blots were hybridized with radiolabeled MYX N1R as probe. Lanes: 1) uninfected; 2) MYX infection (1 hour); 3) MYX infection (2 hour); 4) MYX infection (3 hour); 5) MYX infection (4 hour). Arrows mark the positions of specific early and late transcripts. Molecular size standards (in kilobases) are indicated on the left of the panel.

DISCUSSION

The SFV N1R RING finger protein and several other poxvirus orthologs are known to localize to the virus factory in the cytoplasm of infected cells. In this study I performed deletion and mutational analysis of the SFV N1R RING finger gene and expressed the mutant proteins transiently in VV infected cells to determine which regions of this protein are required for localization to the virus factory. These experiments showed that part of the RING motif region is required for localization but that a substantial portion could be deleted with little or no effect. Another deletion mutant demonstrated that the C-terminal half of the protein that contains the complete RING motif region does not localize to virus factories. In addition, a five amino acid region was recognized at the N-terminus of the SFV protein (amino acids #24-28) that is highly conserved among the poxvirus orthologs and was found to be essential for virus factory localization.

It is therefore apparent that the RING motif is not solely responsible for localization, nor is the entire motif required for this effect. This is consistent with a growing body of evidence that suggests the RING motif does not function as an independent protein domain but may be part of a larger domain of those proteins in which it is found (Bellon *et al.*, 1997; Clem and Miller, 1994; Everett *et al.*, 1995b; Maul and Everett, 1994). A novel finding was the observation that the RING motif of HSV-1 ICP0 could substitute for that of SFV N1R for virus factory localization. This suggests certain RING finger motifs may be interchangeable and as such share a common function. Indeed, recent studies indicate RING fingers may be central to the process of ubiquitination (Freemont, 2000). Other motif switching experiments, however, have not been as successful. The RING motif of PML does not replace that of HSV-1 ICP0 (Everett *et al.*, 1995c) and the EHV RING motif only partially complements the HSV-1 counterpart (Everett *et al.*, 1995b). It will be interesting to determine if the SFV-HSV-1 fusion we have generated retains other characteristics of the wild type SFV N1R protein.

Although DNA binding has been reported for several proteins containing RING motifs (Bailly *et al.*, 1997; Gong *et al.*, 1997; Hiom and Gellert, 1997; Kanno *et al.*, 1995), it does not appear to be a common characteristic of this group of proteins. Attempts to show DNA binding of the EVp28 protein were inconclusive because of protein insolubility problems (Senkevich *et al.*, 1994; Senkevich *et al.*, 1995). After initial difficulties in solubilizing the SFV N1R from VV-N1R infected cells we were able to show that this protein binds to both ds- and ss-DNA cellulose and that EDTA in the binding buffer inhibits this. Two features, the broad range of NaCl concentration over which the SFV N1R eluted from DNA-cellulose (0.5-0.75M NaCl) and its molecular weight, suggest that SFV N1R may be the ortholog of a previously described VV 28 kDa virus factory associated DNA-binding protein (designated FP14) (Nowakowski *et al.*, 1978). However, these experiments were reportedly performed with VV strain WR and it has been shown that VV WR produces a significantly truncated protein that does not localize to the virus factory (Upton *et al.*, 1994). Clarification of this issue must await further experimentation. Studies using the EVp28 mutant indicated that this gene product was required for viral replication in peritoneal resident macrophages because virus factories were not detected by Hoechst dye staining (Senkevich *et al.*, 1995). Thus, the DNA-binding activity of this group of poxviral proteins may only be required as an accessory factor for DNA replication or transcription in certain cell types. Alternatively, these proteins may serve to recruit a host protein to the virus factory.

Structural prediction studies inferred the amino terminal region of N1R likely formed a ribbon-helix-helix DNA interaction motif. Site-specific mutagenesis of the conserved β strand region of N1R provided experimental evidence for this hypothesis. Following transient expression the localization of five N1R mutants each having a potential β strand residue replaced with Ala were characterized. By comparison with members of the ribbon-helix-helix family, Tyr-24 should be the first solvent exposed residue of the β strand. Mutation of Tyr-24 to Ala had a small effect on the localization of N1R to the DNA

containing factories, indicating the Ala residue is compatible with localization. Interestingly, the corresponding His residue in the Mnt repressor when mutated to Ala has almost no effect on the affinity of this Mnt repressor mutant for operator DNA (Waldburger and Sauer, 1995).

Asn-26 should be the second solvent exposed residue of the N1R β strand. This invariant Asn is absolutely conserved among all poxviral orthologs of this gene and interestingly this central β strand solvent exposed residue is also conserved among the Arc and Mnt repressors. Mutation of Asn-26 diminished the localization of N1R to the virus factories and mutant N1RAsn26ΔAla protein in contrast to N1R wildtype failed to interact with bind DNA cellulose. The function of Asn-26 in DNA binding and thus factory localization of N1R is consistent both with the observed tertiary structure of Arc where Asn-11 makes an extensive set of DNA base contacts (Raumann *et al.*, 1994) and mutagenesis studies of Mnt where Asn-8 has been shown to be a major determinant of operator DNA binding (Knight and Sauer, 1992).

This role of Asn in DNA binding proteins is not without precedence. The polar Asn residue has one of the highest propensities for interaction with DNA (Jones *et al.*, 1999; Lustig and Jernigan, 1995), and is critical for DNA binding and specificity in a variety of proteins. Examples include the POU specific homeodomain of Oct-1(Botfield *et al.*, 1994; Pomerantz and Sharp, 1994), restriction endonucleases *Eco*R1 and *Eco*RV (Fritz *et al.*, 1998), DNA repair enzymes such as uracil DNA glycosylase (Mol *et al.*, 1995) and endonuclease HAP1 (Rothwell and Hickson, 1996) and the c-myb protooncogene (Saikumar *et al.*, 1990). Asn is well suited to DNA binding; it forms strong bidentate hydrogen bonds preferentially to adenine to which it can donate a hydrogen bond to the N7 and accept a hydrogen bond from the N6 of this base. As such, this essential conserved Asn may well facilitate binding of N1R to the AT rich poxviral genome.

Thr-28 is proposed to be the last solvent exposed residue of the β strand. Mutation of Thr-28 to Ala had little effect on the localization of N1R to the virus factories. Previously,

a potential consensus N-glycosylation site within N1R corresponding to the sequence Asn-X-Ser/Thr of the proposed β-strand region was identified, however, it seems unlikely that this site is glycosylated because of the cytosolic location of the protein. The fact that the mutation of Thr-28 to Ala destroys the potential glycosylation site but does not alter the molecular weight of the protein also suggests that it is not glycosylated.

Immunolocalization studies have also indicated a potential role for Ile-27 in factory localization and consequently DNA binding, since mutation of this residue to Ala resulted in significantly less factory localization, with smaller defined foci observed. Although this hydrophobic residue is expected to be non-solvent exposed, it is noteworthy that recent studies have identified a critical role for the Phe-10 side chain of Arc, which, although part of the hydrophobic core of the free protein, rotates out to make important sugar-phosphate operator DNA contacts in the repressor operator complex (Schildbach *et al.*, 1999). While Ile is known to make hydrophobic interactions with the methyl group of thymine, it is also possible, from the proximity of this residue to Asn-26, that side chain interactions between Ile-27 and other buried residues of N1R stabilize the Asn-26 in such a steric conformation that favorable interactions with DNA are achieved. In support of this, mutation of the corresponding Leu-12 residue of Arc to Ala is not compatible with a native folded Arc structure (Bowie and Sauer, 1990).

It is possible that the above observed requirement of each of these N-terminal residues in N1R, particularly Asn-26, results from loss of structural integrity of the mutant proteins, however, alanine is least likely to perturb structure and many proteins display significant tolerance to amino acid substitutions (Bowie and Sauer, 1990; Brown and Sauer, 1999). Although this N-terminal region is highly conserved the mutant proteins were expressed at levels comparable to normal N1R. In this respect, recent mutagenesis studies of Arc utilizing a mutant that contained multiple Ala substitutions at the N-terminal region of Arc, encompassing the β–strand and including the corresponding N1R Asn26 residue

Asn-11, showed that although this mutant was capable of adopting a native Arc structure, DNA binding activity was inhibited (Brown and Sauer, 1999).

Although initial deletion mutational analysis identified a short region of the zinc binding RING finger that was necessary to permit normal localization of N1R to the virus factories and consequently implicated the RING finger motif in DNA binding, it is possible that the RING finger region may be required to provide structural integrity to the protein as a whole. As point mutations are less likely to affect structure compared to deletion mutation analysis, these results indicate that the five amino acid β strand region of N1R governs DNA binding activity and thus localization to the virus factories. This conclusion is strongly supported by the fact that a number of individual point mutations within the central region of the RING finger of SFV-N1R had no effect on localization and a chimeric SFV-N1R which contained the RING finger region of HSV type 1 ICPO in place of the RING finger of SFV-N1R localized to the virus factories within the cytoplasm of VV infected cells normally.

Importantly, these results also suggest that the RING finger does not necessarily function similarly to the classical zinc finger DNA binding motif in promoting sequence specific DNA binding (Pabo and Sauer, 1992). In support of this, studies which implicated the RING finger region of the human mel-18 tumor suppresser protein in specific DNA binding relied solely on a deletion of a large region encompassing the RING finger of this protein (Tagawa *et al.*, 1990) and isolated RING finger peptides from the Herpes simplex virus type 1 ICPO protein family and the tumor suppresser BRCA-1 do not bind DNA (Elser *et al.*, 1997; Everett *et al.*, 1993). Also, the proposed DNA-binding domains of lymphoid specific RAG1 protein, mammalian helicase-like transcription factor (HTLF) and the human SNF2/SW12 related HIP116 transcription factor are distinct from the RING finger domains of these proteins (Difilippantonio *et al.*, 1996; Gong *et al.*, 1997; Li *et al.*, 1992; Sheridan *et al.*, 1995; Spanopoulou *et al.*, 1996).

However, this conserved poxviral N-terminal region encompassing Asn-26 is not part of an independent domain because the VV-WR ortholog, which is truncated but contains the complete N-terminal two thirds of the protein, also fails to localize to the factories (Upton *et al.*, 1994). Similar to the Mnt repressor, which contains two independent domains that are both required for high affinity DNA binding (Waldburger and Sauer, 1995), we envisage the RING finger may form a motif, which by itself has no DNA-binding activity, yet through protein-protein interactions helps stabilize the overall protein structure. This study has indicated caution concerning the interpretation of RING finger studies based on deletion mutation analysis or those centered on conserved Cys substitutions within the RING finger that likely abrogate structural integrity of the entire protein. It is likely that the specificity for biological protein activity may not depend solely on the integrity of RING fingers in those proteins containing this motif.

The basic architectural unit of the ribbon-helix-helix family of proteins is that of a homodimer, whereby the β strand of each monomer pairs to form the anti-parallel β ribbon which inserts into and makes base specific contacts with the major groove of DNA. While the active repressor form of MetJ, Arc and Mnt may result from assembly of dimers into fully active tetramers, the active form of TraY is a monomer in solution (Lum and Schildbach, 1999) and the oligomeric nature of AlgZ is unknown. The broad elution profile of N1R from DNA cellulose may indicate oligomer formation with various affinities for the substrate or possibly differential interaction of N1R with diverse local structural forms of the DNA cellulose. Currently, the true oligomeric nature of N1R remains to be determined.

CHAPTER 2: Identification of a Role For the Poxviral RING Finger Proteins Shope Fibroma Virus N1R and Ectromelia Virus p28 in Apoptosis Inhibition.

INTRODUCTION

The recognition of the importance of apoptosis has been one of the most significant changes in the biomedical sciences in the last decade. Although the molecular processes controlling and executing cell death through apoptosis have begun to be defined, the picture is far from complete. Apoptosis is a morphologically distinct form of cell death in metazoan and protozoan organisms. It is now recognized to occur in embryonic development, during normal immune cell proliferation and responses, and as an integral part of tissue homeostasis (Jacobson *et al.*, 1997). Since cells actively take part in the process, apoptosis has been likened to cell suicide. Apoptosis is the process by which a cell is killed and dismantled after receiving one of a variety of death signals, including cellular stresses such as DNA damage, ionizing or UV radiation, heat shock or hypoxia (Hale *et al.*, 1996). Members of the tumor necrosis factor (TNF) family of membrane anchored and secreted ligands acting through cell surface receptors, also play important roles in apoptosis (Ware *et al.*, 1996).

The multistep process of apoptosis is usually characterized by widespread membrane blebbing, cell shrinkage, chromatin condensation and extensive DNA fragmentation (Kerr *et al.*, 1972), but apoptosis can also occur in enucleated cells (Vaux and Strasser, 1996). A key feature of apoptosis is the lack of an inflammatory response. Changes that occur in the plasma membrane result in the recognition and phagocytosis of apoptotic cells which helps prevent an inflammatory response (Nicholson and Thornberry, 1997). Dysfunctional apoptosis is associated with a number of serious diseases and

disorders including neurodegenerative disorders, AIDS and cancer (Thompson, 1995), therefore, the molecular apoptotic pathways are important to define.

Executioners of Apoptosis - Caspases

Initial insight into the molecules executing cell death came from genetic analysis of the nematode *Caenorhabditis elegans* (*C. elegans*). In this worm, 131 cells of the organism's 1090 cells die by apoptosis during normal development (Yuan and Horvitz, 1990). Two genes, ced-3 and ced-4, are essential for apoptosis in *C. elegans* , and the ced-9 gene prevents cell death (Hengartner *et al.*, 1992). Molecular cloning revealed that ced-3 is a homolog of the mammalian interleukin-1β converting enzyme (ICE, now designated Caspase-1) and suggested that these enzymes, conserved between nematodes and mammals, were central to the apoptotic response (Yuan *et al.*, 1993). Indeed, overexpression of ICE induced apoptosis, whereas apoptosis was inhibited by the cowpox serine protease inhibitor CrmA that blocks ICE activity (Tewari and Dixit, 1995). A further thirteen cellular ICE-like proteases have been identified, and this family of cysteine proteases which mediate the specific proteolytic cleavage events in dying cells have been termed caspases (cysteinyl aspartate-specific proteinases), to denote their specificity for cleavage C-terminal to aspartate residues (Alnemri *et al.*, 1996).

Although the morphological changes which occur in cells undergoing apoptosis might suggest widespread proteolysis of cellular constituents, only a discrete number of specific proteins appear to be targeted for proteolytic cleavage once the cell death pathway has been initiated (Nicholson and Thornberry, 1997). In each case, proteolysis occurs after an Asp residue providing compelling evidence for the role of caspases (Nicholson and Thornberry, 1997).

Caspases are synthesized as inactive proenzymes, composed of an N-terminal prodomain, and two domains that are usually separated by a linker region (Nicholson and Thornberry, 1997; Zhivotovsky *et al.*, 1997). They are activated by cleavage after specific aspartate residues (Zhivotovsky *et al.*, 1997). Caspase-1 is synthesized as a 45 kDa cytoplasmic proenzyme that is proteolytically activated by other caspases or autocatalytically, in response to inflammatory signals (Thornberry *et al.*, 1992). The active form is composed of a dimer of a heterodimer, which is composed of 20 kDa and 10 kDa subunits, both derived from the 45 kDa proenzyme (Walker *et al.*, 1994). Caspase-1 recognizes the sequence Tyr-Val-Ala-Asp (YVAD) in proIL-1β and cleaves between Asp116-Ala117 to generate the mature, biologically active form of this cytokine (Walker *et al.*, 1994). In addition, caspase-1 has been recently implicated in the maturation of interferon-γ inducing factor (IGIF), an 18 kDa cytokine that stimulates T-cell production of interferon–γ (Ghayur *et al.*, 1997).

The crystal structure of caspase-1 complexed with specific peptide inhibitors has been determined (Walker *et al.*, 1994; Wilson *et al.*, 1994). The active enzyme is a tetramer of two p20 subunits and two p10 subunits. The complex is stabilized primarily by contacts between the p10 subunits although interactions between the p20 C-terminus and the p10 N-terminus are also important (Walker *et al.*, 1994). The active site is composed of residues from both the p20 and p10 subunits. The active site cysteine is essential and occurs in the conserved pentapeptide sequence QACRG of the p20 subunit (Walker *et al.*, 1994). The catalytic machinery involves a dyad composed of a cysteine sulphydryl group (Cys285) in close proximity to a histidine imidazole group (His 237) on the larger subunit (Wilson *et al.*, 1994). Using a mechanism similar to other cysteine proteases, these enzymes appear to stabilize the oxyanion of the tetrahedral transition state through hydrogen bonding interactions with the backbone amide protons of Cys 285 and Gly 238 (Wilson *et al.*,

1994). Additionally, four residues, two from each subunit, appear to be involved in stabilization of the substrate P_1 Asp (Arg 179, Gln283, Arg341, Ser 347) (Wilson *et al.*, 1994). The two arginine residues (Arg-179 and Arg-341) form hydrogen bonds with the P1 Asp and mutation of these residues result in the loss of protease activity. Side chains of residues of p10 from Val-338 to Pro-343 interact with P2-P4 sites of the tetrapeptide-aldehyde inhibitor (Wilson *et al.*, 1994). It is proposed that maturation of the proenzyme occurs when two precursor p45 proteins associate and are processed. The p10 subunit from one caspase-1 molecule probably complexes with the p20 subunit of another caspase-1 molecule to create the active site (Walker *et al.*, 1994). The prodomain of caspase-1 may have a regulatory role in this process since it is absolutely required for dimerization and autoproteolysis (Fraser and Evan, 1996).

Phylogenetically, caspases have been divided into 3 subfamilies: an ICE subfamily (caspases-1,-4 and -5), a CED3/CPP32 subfamily (caspases-3, -6, -7, -8, -9 and -10), and an ICH-1 subfamily (caspase-2) (Alnemri *et al.*, 1996). Caspase-3 (32 kDa) is one of the key executioners of apoptosis. It is responsible for the proteolysis of a large number of substrates, each of which contains a common Asp-Xaa-Xaa-Asp (DXXD) motif (Tewari *et al.*, 1995b). In contrast to caspase-1, caspase-3 has no linker peptide and the prodomain is much smaller (Rotonda *et al.*, 1996). The crystal structure of caspase-3 in association with a tetrapeptide aldehyde inhibitor revealed an overall similar structure that is similar to caspase-1. The S4 subsite, however, is very different in size and chemical composition. This accounts for differences in specificity (Rotonda *et al.*, 1996). The S4 subsite of caspase-1 is a large shallow hydrophobic depression that readily accommodates a tyrosyl side chain whereas this site in caspase-3 is a narrow pocket that closely surrounds the P4 Asp side chain (Rotonda *et al.*, 1996).

Important targets of caspase-3 activity include the DNA repair enzymes poly (ADP-ribose) polymerase (PARP) and DNA dependent protein kinase (Tewari *et al.*, 1995b); the cell cycle regulatory protein, retinoblastoma protein (Rb) (Dou *et al.*, 1997); the negative regulator of p53 activity, mdm2 (Chen *et al.*, 1997; Thut *et al.*, 1997); protein kinase C (Ghayur *et al.*, 1996) and the inhibitor of caspase-activated DNase (ICAD, also known as DFF45) (Enari *et al.*, 1998; Liu *et al.*, 1997). Most caspase substrates are inactivated by proteolysis, but caspase-3 cleavage of protein kinase C, ICAD and the actin modulating protein gelsolin, activates their role in promoting apoptosis (Kothakota *et al.*, 1997; Liu *et al.*, 1997). Caspase-3 is directly activated by granzyme B during T- cell mediated cytotoxicity (Quan *et al.*, 1996). Active caspase-3 can cleave pro-caspase-6, which is also able to activate pro-caspase-3, thus setting up a protease amplification cycle (Fernandes-Alnemri *et al.*, 1996). Some caspase substrates are cleaved by more that one caspase family member (caspase-3 and caspase-7 can both cleave PARP), whereas others may be targets for a single caspase (caspase-6 is the only caspase known to cleave lamins) (Orth *et al.*, 1996). In addition, alternatively spliced isoforms are produced from some caspase mRNAs. These may regulate the activity of the full length enzymes (Scaffidi *et al.*, 1997).

Caspase substrates encompass catalytic and structural proteins involved in homeostasis (PARP, DNA-PK), splicing (U1-70 kDa) (Tewari *et al.*, 1995a), cellular signaling (PKC), cell cycle control or tumor suppression (mdm2, Rb), and cellular architecture (lamins) (Rotonda *et al.*, 1996). Proteolysis of these substrates leads to detachment and disassembly of the cell and its disposal by immune cells such as macrophages. Caspase knock-out mice have complex phenotypes, but it is thought that multiple mechanisms of caspase activation exist and that death signal transduction pathways are both cell-type and stimulus specific (Hakem *et al.*, 1998; Kuida *et al.*, 1998; Kuida *et al.*, 1996). Thus, for a given death signal, a specific caspase may be essential for apoptosis in one cell type but dispensable in another. Caspase-9 deficient ES cells are resistant to

exposure to UV and γ-radiation, whereas thymocytes from the same knockout mice are resistant to γ-radiation but sensitive to UV irradiation (Hakem *et al.*, 1998; Kuida *et al.*, 1998).

Caspases may also contribute to diverse developmental phenomena. For example, mice lacking caspase-8 have an abnormality in heart development indicating a role for caspase-8 in cardiomyogenesis. It is unclear, however, whether this requirement for caspase-8 is directly related to apoptosis (Varfolomeev *et al.*, 1998).

Cytokines and Receptor Mediated Apoptosis

Apoptosis signaling frequently initiates at the cell surface upon interaction of specific ligands with their cognate receptors (Smith *et al.*, 1994). Fas ligand (FasL) and tumor necrosis factor (TNF) are the best characterized death factors. They bind to their receptors and induce apoptosis, killing the cells within hours (Cleveland and Ihle, 1995). FasL and TNF belong to the TNF family, which includes lymphotoxin, CD30 ligand, 4-1BB ligand, CD40 ligand, CD27 ligand, TRAMP and TRAIL (Nagata and Golstein, 1995; Pan *et al.*, 1997; Sheridan *et al.*, 1997).

FasL is predominantly expressed in activated T-lymphocytes and natural killer (NK) cells, but is also constitutively expressed in the tissues of "immune-privilege sites" such as the testis and eye (Griffith *et al.*, 1995). FasL induced cell death is required for the normal elimination of potentially autoreactive peripheral T-cells, as well as being central to the induction of T-cell mediated cytotoxicity (Dhein *et al.*, 1995). This is evidenced by the phenotype of mice homozygous for the *lpr* or *gld* mutations (Fas and FasL respectively), which develop a fatal lymphoproliferative disease (Gillette-Ferguson and Sidman, 1994).

The central role of Fas is to trigger apoptosis, whereas TNF is a pleiotrophic cytokine produced by macrophages, T cells and nonlymphoid cells that can induce differentiation, proliferation or cell death (Ware *et al.*, 1996). This diversity of biological function stems in part from its ability to interact with two distinct cell surface receptors, TNFR1 (55 kDa) and TNFR2 (75 kDa), that are expressed at varying levels on most cells (Rothe *et al.*, 1994). Signaling through TNFR1 is associated with most of the TNF induced effects, including cytotoxicity in diverse cell types, but TNFR2 stimulation has been associated with cell proliferation through the activation of NFkB (Ware *et al.*, 1996).

FasL and TNF are type II -membrane proteins; their N-termini are located in the cytoplasm and the C-terminal domains extend into the extracellular space. This extracellular domain, approximately 150 amino acids, is well conserved (20-25%) among members of the TNF family, but the lengths and sequence of the cytoplasmic domains differ significantly (Nagata and Golstein, 1995). Proteolysis of membrane bound FasL or TNF by a membrane associated metalloproteinase produces soluble ligand, however, this process significantly attenuates function of the ligands, suggesting that the ligands act locally via cell-cell interactions (Nagata, 1997). Indeed, membrane bound TNF is more active than soluble TNF in activating the type II TNF receptor (Nagata, 1997). FasL and TNF mediate their effects through interaction with structurally related type I membrane receptors belonging to the TNFR superfamily (Armitage, 1994; Smith *et al.*, 1994). Most of the receptors in this family recognize ligands that are costimulators of the immune response rather than inducing cell death (Chinnaiyan and Dixit, 1997; Cleveland and Ihle, 1995). The TNFR superfamily includes Fas (Apo-1/CD95), the receptor for FasL, two TNFRs (TNFR1 and TNFR2), DR3, TRAILR, the receptor for lymphotoxin-β, nerve growth factor receptor (NGFR), CD40, CD27 and CD30 (Chinnaiyan *et al.*, 1996a; Pan *et al.*, 1997; Sheridan *et al.*, 1997; Smith *et al.*, 1994). The extracellular ligand binding region of these receptors possess 2-6 repeats of a cysteine rich subdomain that has 20-30% identity among the various family

members (Chinnaiyan *et al.*, 1996a). Crystallographic analysis of a soluble TNFR1 complexed with TNF showed that three receptors bind one TNF trimer (Banner *et al.*, 1993). Receptor aggregation mediated by the respective ligands FasL and TNF directly couples the receptors to the death pathway (Boldin *et al.*, 1995a). The cytoplasmic regions lack intrinsic kinase activity and there is little similarity among family members, except for Fas, TNFR1, DR3 and DR4 which share homology over a 60-80 amino acid region that is distantly related to the *Drosophila* suicide gene reaper (Baker and Reddy, 1996; Golstein *et al.*, 1995). Mutational analysis of this region in Fas and TNFR1 has shown that this "death domain" region is responsible for transducing the death signal (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). This domain, which has a propensity to self-aggregate, has a novel structure consisting of six antiparallel amphipathic α helices, with a large number of surface charged residues (Huang *et al.*, 1996). It is believed that this allows for interaction with adapter proteins containing a similar death domain (Huang *et al.*, 1996).

Utilization of the yeast two hybrid system with the Fas cytoplasmic domain as bait led to the identification of FADD-1 (also known as MORT-1) that contains a death domain at its C-terminus (Chinnaiyan *et al.*, 1995). FADD is recruited and binds to activated Fas through interactions between the death domains (Boldin *et al.*, 1995b). The N-terminal region of FADD, which has been termed the death effector domain (DED), is both necessary and sufficient to engage the downstream death-signaling pathway (Hsu *et al.*, 1996b). Similarly, TRADD (TNFR-associated death domain) was found to bind TNFR1 (Hsu *et al.*, 1995), however, TRADD lacks a death effector domain. The finding that TRADD binds to FADD via interactions between their death domains suggests that both Fas and TNFR1 use FADD as a common signal transducer and share the signaling machinery downstream of FADD/MORT1 (Hsu *et al.*, 1996b). In this respect, a dominant negative version of FADD (FADD-DN) blocks TNF-and Fas induced apoptosis (Chinnaiyan *et al.*, 1996b).

Two groups independently identified the signaling molecule downstream of FADD/MORT1 (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Using the N-terminal DED of FADD/MORT1 as bait in a yeast two-hybrid screen, Boldin *et al* identified FLICE, whereas biochemical characterization of molecules recruited to Fas upon ligand activation identified MACH (Muzio *et al.*, 1996). FLICE/MACH interacts with FADD/MORT to form a death inducing signaling complex (DISC) (Kischkel *et al.*, 1995). FLICE/MACH is a member of the caspase family (now designated caspase-8), (Alnemri *et al.*, 1996) providing a direct link between ligand induced TNFR activation and the caspases. Caspase-8 carries two DED domains at its N-terminus (prodomain) through which it binds FADD/MORT1 (Boldin *et al.*, 1996; Muzio *et al.*, 1996). A dominant negative mutant of caspase-8, in which the active site cysteine is altered, blocks both Fas and TNF-induced apoptosis.

Upon Fas receptor triggering, caspase-8 is recruited to the DISC where it is proteolytically activated (Medema *et al.*, 1997). It is currently unknown how aggregation of the signaling components leads to activation of caspase-8, though interactions between the DED homology regions of caspase-8 and FADD/MORT1 may allow for autocatalytic cleavage (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Caspase-8 exists in multiple isoforms that may indicate possible control mechanisms (Fraser and Evan, 1996). Recombinant caspase-8 is able to activate a variety of caspases suggesting that it lies at the apex of a proteolytic apoptotic cascade (Srinivasula *et al.*, 1996), at least with respect to FasL or TNF triggered apoptosis.

The TNFR family members can also recruit a second class of signal transducers. The TRAF (TNF receptor-associated factor) family which has 6 members (Inoue *et al.*, 2000), are characterized by the presence of a conserved C-terminal 230 amino acid TRAF domain that mediates oligomerisation among family members and an N-terminal region

containing a RING finger motif (Lee and Choi, 1997). TRAFs mediate NFkB activation induced by TNF, CD30L, CD40L and interleukin-1 (Cao *et al.*, 1996; Cheng *et al.*, 1995). TRAF2 binds directly to TNFR2, CD30 and CD40 and indirectly to TNFR1 through TRADD and RIP (Darnay and Aggarwal, 1997; Hsu *et al.*, 1996a). A dominant negative TRAF2 blocks TNF-induced NFkB activation, but not apoptosis (Hsu *et al.*, 1996b) indicating that the signaling pathways are distinct.

NFkB consists of two subunits (p50 and p65) and exists in a complex with IkB in resting cells. The crucial step for NFkB activation is the phosphorylation of IkB, leading to its ubiquitination and subsequent degradation by the proteosome (Karin, 1999). This phosphorylation is catalyzed by a TRAF2 associated NFkB inducing kinase (NIK) (Nakano *et al.*, 1998). NFkB, thus released from IkB, enters the nucleus and activates genes possessing the NFkB response element (Finco and Baldwin, 1995). Surprisingly, disruption of the NFkB pathway enhances the cytotoxic effects of TNF. This suggests the presence of downstream genes that mediate protective survival functions (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996).

The mammalian inhibitors of apoptosis (IAPs) are one such emerging family of protective genes (Uren *et al.*, 1996). Conserved in organisms ranging from insects to humans, this family of homologs of the baculovirus IAP protein (Crook *et al.*, 1993) notably includes the candidate gene for spinal muscular atrophy (Liston *et al.*, 1996). Human c-IAP2 is under NFkB control. It associates with TRAF2 and suppresses TNF cytotoxicity by upregulating NFkB activity, but the precise mechanism by which this occurs remains to be determined (Chu *et al.*, 1997).

Mitochondrial Regulation of Apoptosis

What has recently become increasingly clear is the importance of mitochondria in apoptosis regulation (Green and Reed, 1998). Mitochondria act as critical sensors and amplifiers in intracellular death signaling pathways (Susin *et al.*, 1998). Mitochondrial damage, observed as swelling, disruption of the outer membrane, depolarization, and the release of the pro-apoptotic factors cytochrome C and the oxidoreductase related flavoprotein, apoptosis inducing factor (AIF) (Kluck *et al.*, 1997; Susin *et al.*, 1999; Susin *et al.*, 1996; Vaux and Strasser, 1996) is frequently evident during early apoptosis. A large number of diverse death stimuli trigger mitochondrial damage and the critical release of cytochrome c into the cytosol (Green, 1998). After cytochrome c is released from mitochondria it binds to a 130 kDa ubiquitously expressed human cytosolic ced-4 homolog termed Apaf-1 (apoptosis protease activating factor-1), and through association with procaspase 9 activates the caspase cascade (Li *et al.*, 1997; Yang *et al.*, 1997; Zou *et al.*, 1997). This critical apoptotic signaling complex, termed the apoptosome (Zou *et al.*, 1999), is regulated by the Bcl-2 protein family and constitutes a major life or death decision point at the mitochondria (Tsujimoto and Shimizu, 2000; White, 1996). In excess, Bcl-2 can inhibit the release in cytochrome c whereas caspase inhibitors do not. This indicates that cytochrome c release and activation of Apaf-1 are downstream of Bcl-2 function but upstream of the caspases (Kluck *et al.*, 1997).

Bcl-2 was first identified in B-cell follicular lymphomas where it is overexpressed as a result of a t(14:18) chromosomal translocation (Tsujimoto *et al.*, 1985). Bcl-2 is homologous to ced-9 and can functionally replace it, preventing cell death in *C. elegans* (Hengartner and Horvitz, 1994). Overexpression of Bcl-2 blocks apoptosis of mammalian cells that is triggered by a number of stimuli such as growth factor withdrawal, UV-A and UV-B irradiation (Muller-Rover *et al.*, 2000; Suschek *et al.*, 1999), c-myc or anti-cancer

drugs (Chiou *et al.*, 1994; Miyashita *et al.*, 1997; White, 1996). A large number of Bcl-2 family proteins have been described which include both pro- and anti-apoptotic members (Antonsson and Martinou, 2000; Hawkins and Vaux, 1997). The Bcl-2 subfamily, including Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1 are potent suppressers of apoptosis (Tsujimoto and Shimizu, 2000). These proteins are characterized by up to four short sequences, termed Bcl-2 homology (BH1–4) domains, and a membrane anchor that directs them to endoplasmic reticulum, mitochondrial, and nuclear membranes (Reed *et al.*, 1996). Proapoptotic family members such as Bax, Bak, Bad, Bcl-X_S, Bik, Bid and Bim all contain an essential proapoptotic BH3 domain (Antonsson and Martinou, 2000; Kelekar and Thompson, 1998).

BH domains function in both homomeric and heteromeric binding, both between anti and pro-apoptotic Bcl-2 family members as well as in interactions with other regulatory proteins, which is considered to constitute one mechanism of regulating their biological activity (Reed *et al.*, 1996; Zha *et al.*, 1997). The ratio of inhibitors to activators within the cell may determine whether or not the cell undergoes apoptosis (Perlman *et al.*, 1999). For example, when high levels of Bcl-2 dimerize with Bax, the death effect of Bax is neutralized (Gajewski and Thompson, 1996). Further, mutations in the BH3 domains of various pro-apoptotic proteins that disrupt the interaction between these proteins and Bcl-2 can also markedly impair their death-inducing activities (Chittenden *et al.*, 1995; Wang *et al.*, 1998; Wang *et al.*, 1996). Individual members, however, act with different potencies and with different lineage specificity (Hockenbery, 1995; Hsu and Hsueh, 2000).

The initial insight into the molecular apoptotic function of the Bcl-2 family came from determination of the crystal structure of the Bcl-xL protein. It revealed two central hydrophobic α helices similar to those in the pore-forming diphtheria toxin and the colicins (Muchmore *et al.*, 1996). Subsequently, Bcl-2 family members such as Bcl-2, Bcl-X_L, Bax and Bid were found to form ion channels in synthetic lipid membranes (Minn *et al.*, 1997;

Schendel *et al.*, 1999; Schlesinger *et al.*, 1997) suggesting a possible mechanism for their action. Both Bcl-2 and Bcl-X_L have been shown to prevent cytochrome c release from mitochondria (Manon *et al.*, 1997; Yang *et al.*, 1997). Further, addition of recombinant proapoptotic Bax or Bak to isolated mitochondria induces cytochrome C release (Jurgensmeier *et al.*, 1998; Shimizu *et al.*, 1999). The proapoptotic effects of Bax may be elicited through an intrinsic pore forming activity that can be agonized by Bcl-2 (Antonsson *et al.*, 1997). This may explain the membrane permeability transition (PT) that occurs in apoptosis and the ability of Bcl-2 family members to regulate it (Marchetti *et al.*, 1996).

The PT pore is an oligo-protein channel consisting of the voltage dependent anion channel (VDAC) on the outer membrane, adenine nucleotide translocator (ANT) on the inner membrane and matrix protein cyclophilin D (Marzo *et al.*, 1998b). Bax interacts with VDAC and ANT (Marzo *et al.*, 1998a; Narita *et al.*, 1998). Both Bax and Bak have been shown to promote opening of this channel (Shimizu *et al.*, 1999), whereas the antiapoptotic Bcl-X_L closes it (Vander Heiden *et al.*, 1999). Thus Bcl-2 family members likely regulate cytochrome C release through modulation of the permeability of the VDAC.

Bcl-2 family members are regulated by a variety of mechanisms such as proteolysis, translocation, transcription and post-translational modifications (Korsmeyer, 1999). The mammalian Bid protein which is a specific proximal substrate of caspase-8 in the Fas signaling pathway (Li *et al.*, 1998; Luo *et al.*, 1998) lacks a transmembrane domain and is cytosolic. However, after cleavage by caspase-8, Bid translocates from the cytosol to the mitochondrial membrane, and thereby mediates transmission of the death signal from the cell membrane to the mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998). Bax is also upregulated during excito- and geno-toxicity induced apoptosis of cortical neurons in a p53-dependent manner (Xiang *et al.*, 1998); and Bax moves from the cytosol to

mitochondria in response to cell stimulation with death-inducing agents such as staurosporine (Wolter *et al.*, 1997).

Certain survival factors, such as insulin-like growth factor-1 (IGF-1), activate the protein kinases Akt and PDK-1 via the Ras-phosphatidyl inositol 3' kinase pathway resulting in phosphorylation and inactivation of proapoptotic Bad (del Peso *et al.*, 1997). Phosphorylated Bad loses its ability to bind Bcl-xL and becomes bound to cytosolic 14-3-3, a specific phosphoserine binding protein (Zha *et al.*, 1997). Sequestration of Bad by phosphorylation may allow Bcl-xL to function in an anti-apoptotic mechanism (Zha *et al.*, 1997). Akt is also found to phosphorylate and block the activation of procaspase 9 (Cardone *et al.*, 1998). These processes are believed to negatively regulate apoptosis and permit normal cell cycling in the absence of apoptotic signals.

DNA damage induced apoptotic responses

In response to DNA damage and replication interference, cells activate signal transduction pathways that prevent cell cycle progression and induce the transcription of genes that facilitate DNA repair (Elledge, 1996). Arrest in G1/S is thought to prevent replication of damaged templates and arrest at G/2M prevents the segregation of defective chromosomes to daughter cells (Elledge, 1996). In the event of irreparable DNA damage, cells respond with the induction of apoptosis, as a mechanism to destroy and prevent the continuation of the damaged cell within the organism (Kharbanda *et al.*, 1997). The intracellular signals upstream of caspase activation that control this process are fragmented and unclear. Depending on the DNA damaging agent used in an experiment, a diverse array of DNA damage signaling and gene induction responses are elicited upstream of caspase activation (Shaulian and Karin, 1999).

Primary among mammalian checkpoint genes are the tumor suppresser genes ATM, and p53 (Elledge, 1996). The ATM gene is mutated in patients with ataxia telangiectasia, a fatal disease characterized by immunological impairment, gonadal atrophy, hypersensitivity to ionizing radiation leading to cancer predisposition and also ataxia associated with progressive cerebellar Purkinje cell death (Shiloh and Rotman, 1996). ATM and the related mammalian protein Atr (AT and rad-related) are members of a phosphoinositol kinase family that also includes DNA-PK (Cliby *et al.*, 1998; Xu *et al.*, 1998). ATM is specifically required for at least three checkpoints, G1/S, S and G2/M that are activated in response to ionizing radiation (Xu *et al.*, 1998). Following ionizing irradiation, but not UV irradiation, ATM specifically interacts with and phosphorylates a number of substrates including Brca1 (Cortez *et al.*, 1999), p53 (Canman *et al.*, 1998) and the nuclear tyrosine kinase c-Abl (Baskaran *et al.*, 1997; Shafman *et al.*, 1997). Activated c-abl has been shown to phosphorylate p73 (a p53 family member) after γ irradiation, but not following UV irradiation (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999). The proapototic activity of p73 is potentiated by c-abl and diminished in c-abl null cells suggesting specific DNA damage signals are channeled through c-abl, possibly through ATM phosphorylation and thence to p73 in response to γ irradiation (White and Prives, 1999). Loss of ATM slows the induction of p53 protein in response to the DNA strand breaks induced by γ-irradiation, however, ATM mutants die via p-53 dependent apoptosis in response to UV irradiation (Agarwal *et al.*, 1998). This suggests that other upstream components, currently undefined, distinct from ATM and c-abl specifically regulate p53 in response to UV induced signals.

In contrast to the molecular mechanisms involved in receptor mediated apoptosis those involved in DNA damage and particularly UV induced apoptosis remain largely obscure (Martin and Cotter, 1991; Martin *et al.*, 1995). A variety of components such as

protein kinase signaling pathways (stress activated protein kinase/c-jun N-terminal protein kinase, p38 mitogen activated protein kinase and protein kinase C isoforms) (Berra *et al.*, 1997; Denning *et al.*, 1998; Frasch *et al.*, 1998; Zanke *et al.*, 1996), Myc (Sugiyama *et al.*, 1999), Rec2/Rad51B (Havre *et al.*, 1998) and oxidative stress (Verhaegen *et al.*, 1995) have been implicated in the UV response, however, their specific roles remain controversial.

The most prominent effector in the UV response is undoubtedly the cell cycle regulated tumor suppresser protein p53 which acts as a DNA damage sensor (Lane, 1992; Lowe *et al.*, 1993; Yonish-Rouach *et al.*, 1991; Zhan *et al.*, 1993). The inactivation of the p53 gene in more than 50% of human cancers has driven an intense search for the physiological and biological properties of the protein. p53 is a major cell-cycle checkpoint regulator that induces growth arrest following transcriptional activation of the cyclin dependent kinase inhibitor p21 (Elledge, 1996). In addition, p53 promotes apoptosis not only in response to DNA damage such as UV light but also in response to intracellular disruptions resulting from metabolite deprivation, heat shock, hypoxia and activated cellular or viral oncoproteins (Levine, 1997).

While p53 is perhaps the most studied of all proteins, it's apoptotic mechanism(s) is not fully understood. For example, it has remained enigmatic how DNA damage is sensed within the cell, and how p53 receives the activation signal. It seems, however, that multiple pathways of p53 activation and p53-induced apoptosis may exist. UV irradiation of mammalian cells results in a dose dependent increase in p53 levels, largely through protein stabilization mechanisms (Maltzman and Czyzyk, 1984). p53 mediated apoptosis is likely triggered in part through the activation of its downstream genes such as $p21^{Cip1/Waf1}$, GADD45, PAG 608, IGF-BP3 and noticeably Bax, a proapoptotic bcl-2 family member (Buckbinder *et al.*, 1995; Gujuluva *et al.*, 1994; Israeli *et al.*, 1997; Liu and Pelling, 1995;

Zhan *et al.*, 1994). In addition, p53 has been found to downregulate bcl-2 expression (Miyashita *et al.*, 1994).

A novel finding is that p53 induced apoptosis involves transcriptional activation of a number of redox related genes with a concomitant increase in oxidative stress (Polyak *et al.*, 1997). This finding has been recently supported using gene microarray technology analysis (Zhao *et al.*, 2000). According to this model, p53 promoted increases in reactive oxygen species lead to the oxidative degradation of mitochondrial components and activation of the downstream effector caspases (Polyak *et al.*, 1997). p53, however, also induces apoptosis, in an undefined mechanism, independent of its ability to activate transcription (Caelles *et al.*, 1994; Haupt *et al.*, 1995). The critical role of p53 in the response of mammalian cells to UV-induced apoptosis is shown by p53-knockout mice in which epidermal cells undergo reduced apoptosis after UV irradiation (Ziegler *et al.*, 1994). It is interesting to note that p53 is targeted for inactivation by many DNA viruses (Havre *et al.*, 1995; Moore *et al.*, 1996; Okan *et al.*, 1995; Scheffner *et al.*, 1990; Steegenga *et al.*, 1996; Szekely *et al.*, 1993; Wang *et al.*, 1995; Werness *et al.*, 1990), a fact that suggests a p53 anti-viral function.

Poxviruses and Apoptosis

Numerous viruses have evolved genes encoding proteins that effectively modulate apoptosis of the infected cell. Advances in the understanding of the molecular biology of apoptosis has allowed the identification of a myriad of viral apoptosis regulators and, importantly, resulted in a growing awareness of the role of apoptosis in viral infection. Viruses are strict intracellular parasites. Therefore, a successful viral infection resulting from the efficient production and spread of progeny requires evasion of host defense mechanisms that limit virus replication by promoting apoptosis of infected cells (Teodoro

and Branton, 1997). Inhibition of apoptosis has been described for many virus groups and is associated with viral virulence, oncogenesis, latency and persistence (Roulston *et al.*, 1999). Appreciation of the remarkable versatility of viruses comes from the observation that certain viruses that encode gene products that oppose apoptosis, may additionally encode gene products that actively induce apoptosis as part of an exit strategy to facilitate virus spread. In these cases, a delicate balancing act between inhibition and induction of apoptosis is governed by a carefully controlled pattern of viral gene expression.

Poxviruses have evolved a number of strategies to curtail the host's apoptotic response to infection (reviewed in McFadden and Barry, 1998). These include proteins that directly reduce levels of apoptosis inducers such as TNF (Macen *et al.*, 1996b; Schreiber *et al.*, 1997; Sedger and McFadden, 1996), double stranded (ds) RNA (Kibler *et al.*, 1997; Lee and Esteban, 1994; Rivas *et al.*, 1998) and oxidative stress (Shisler *et al.*, 1998). The discovery and analysis of these poxviral proteins has provided critical insights into cellular apoptotic processes, and this trend will undoubtedly continue.

The best known poxviral apoptosis inhibitor is CrmA (SPI-2), which was identified in cowpox virus (CPV) as a protein which inhibits the development of hemorrhagic lesions following infection (Pickup *et al.*, 1986). In contrast to CPV, which produces red hemorrhagic flat lesions on the chicken chorioallantoic membrane, CPV SPI-2 gene mutants produced white raised lesions due to the influx of inflammatory cells into the lesion (Palumbo *et al.*, 1989; Pickup *et al.*, 1986). Sequence analysis identified CrmA as a member of the serine protease inhibitor (serpin) protein family (Pickup *et al.*, 1986). Serpins regulate a number of key cellular biological processes such as fibrinolysis, inflammation and cell migration (Moon *et al.*, 1999). CrmA was found to block the activation of interleukin-1β-converting enzyme (ICE), a protease involved in the processing of interleukin-1β from its inactive precursor to its active form (Ray *et al.*, 1992).

The observation that CrmA expression provided protection from death receptor ligation and cytotoxic T lymphocyte killing (Dobbelstein and Shenk, 1996; Tewari and Dixit, 1995; Tewari *et al.*, 1995c) reinforced a role for ICE in apoptosis execution and promoted the search for other ICE related proteins. ICE (now designated caspase-1), became the prototype for the growing family of proteins, termed caspases (Alnemri *et al.*, 1996), which we recognize today as the critical proteases required for the execution and manifestation of apoptosis. CrmA has been found to be a rather promiscuous protease inhibitor (Zhou *et al.*, 1997). In addition to binding and inhibiting caspase-1 (Ray *et al.*, 1992), it inhibits the activation of caspase-8 (Zhou *et al.*, 1997), the most proximal caspase in the death inducing signaling complex (DISC), caspase-3 (Tewari *et al.*, 1995b) and granzyme B (Quan *et al.*, 1995), the serine proteinase released from granules of cytotoxic T-lymphocytes.

Related serpins are present in a number of poxviruses. MYX encodes a protein closely related to CrmA, termed Serp-2 (Petit *et al.*, 1996), which *in vitro* inhibits caspase-1 (Petit *et al.*, 1996) and granzyme B (Turner *et al.*, 1999). MYX lacking Serp2 has a highly attenuated phenotype in rabbits (Messud-Petit *et al.*, 1998). Whereas MYX infection is associated with blockade of the hosts inflammatory response at the vascular level, rapid inflammatory reactions occurred upon infection with the Serp2 mutant MYX and infected cells from rabbit lymph nodes were found to rapidly undergo apoptosis, suggesting that this gene may increase viral virulence by impairing host inflammatory responses and apoptosis (Messud-Petit *et al.*, 1998). MYX Serp2, however, is unable to functionally substitute for CrmA within the context of cowpox virus infection indicating the protease inhibition profile for Serp2 and CrmA are distinct (Turner *et al.*, 1999). Additionally, MYX encodes Serp1 (Upton *et al.*, 1990), a secreted glycosylated serpin (Macen *et al.*, 1993). Mutation of the Serp1 gene in MYX also results in a significant attenuation of the virus such that more than

50% of infected animals recover from the otherwise lethal infection. Histological analyses of lesions taken from infected animals suggest that in the absence of the SERP1 protein, a more effective inflammatory response occurs. (Macen *et al.*, 1993).

The SPI-2 of rabbitpox (Macen *et al.*, 1996a) and B13R (SPI-2) of VV WR (Dobbelstein and Shenk, 1996)(221), which are both very similar to CrmA can also inhibit FasL and TNF induced apoptosis. Remarkably, however, while B13R inhibits caspase-1 and protects virus-infected cells from TNF and Fas mediated apoptosis, it does not inhibit IL-1β-induced fever (Kettle *et al.*, 1997). Other serpins such as SPI-1 (rabbitpox) and B22R (VV WR) share approximately 45% amino acid identity with CrmA but have different amino acids at their reactive center from those in CrmA and are therefore likely to block different proteases (McFadden and Barry, 1998). Indeed, rabbitpox virus SPI-1 protein has recently been found to form a complex with the chymotrypsin family member cathepsin G, a constituent of neutrophils (Moon *et al.*, 1999). SPI-1 expression is required for the replication of rabbitpox virus (RPV) in PK-15 or A549 cells (Ali *et al.*, 1994). Examination of RPV delta SPI-1-infected A549 cells revealed cellular DNA fragmentation following infection with this mutant RPV, suggesting that the host range defect is associated with the onset of apoptosis (Brooks *et al.*, 1995). Apoptosis was only observed in RPV delta SPI-1 infection of nonpermissive (A549 or PK-15) cells and was absent in wild-type RPV infection (Brooks *et al.*, 1995).

In addition to direct inhibition of caspase activity, poxviruses also encode gene products that act indirectly to inhibit caspase recruitment and subsequent activation. The sequencing of the MCV genome identified a novel mechanism of apoptosis inhibition. This virus encodes proteins that act as intracellular mimics of apoptosis signaling molecules (Bertin *et al.*, 1997). Sequence analysis identified two gene products MC159 and MC160 with significant similarity to the death effector domains (DED) responsible for linking death

receptors, such as Fas and TNFR and the FADD adapter protein to procaspase 8. As expected, these gene products, FLIPs (FLICE inhibitory proteins), were found to bind FADD and inhibit recruitment of procaspase 8 to the receptor complex following FasL or TNF receptor engagement (Bertin *et al.*, 1997).

Additionally, ds RNA which acts as a trigger for interferon induction following viral infection promotes apoptosis of viral infected cells (Kibler *et al.*, 1997; Lee and Esteban, 1994). Interferons upregulate 2'-5' oligoadenylate (2'-5' A) synthetase, an enzyme that synthesizes 2'-5' A. Translation is inhibited when 2'-5'A dependent RNase L is activated and degrades viral and cellular mRNA (Castelli *et al.*, 1997). Second, interferons also induce the synthesis of PKR, a double-strand–RNA-dependent kinase (Lee and Esteban, 1994). When PKR is activated during infection, it phosphorylates the translation initiation factor eIF-2, leading to inhibition of protein synthesis and the induction of apoptosis (Srivastava *et al.*, 1998).

Poxviruses are endowed with mechanisms to protect against intracellular apoptotic signals promoted by IFN. VV encodes at least two proteins that inhibit PKR action. The E3L gene product has a conserved dsRNA binding domain in its carboxyl-terminal region through which it sequesters dsRNA and prevents the interaction of PKR with viral RNA (Chang *et al.*, 1992; Davies *et al.*, 1993; Kibler *et al.*, 1997; Lee and Esteban, 1994). E3L can inhibit apoptosis induced by infection with an E3L mutant virus or by overexpression of activated PKR (Kibler *et al.*, 1997; Lee and Esteban, 1994). E3L can also inhibit apoptosis induced by RNase L and 2'-5'A synthetase overexpression (Rivas *et al.*, 1998). A second vaccinia protein, K3L, is less effective than E3L in inhibiting PKR, and, because it resembles eIF-2, it may interfere with PKR–eIF-2 interactions (Davies *et al.*, 1993).

In addition to blocking intracellular apoptotic signals some poxvirus proteins function extracellularly. The T2 genes of SFV and MYX encode secreted proteins with similarity to the N-terminal ligand binding domains of the cellular TNF receptors (Smith *et al.*, 1991; Upton *et al.*, 1991). MYX T2 protein is secreted early during infection and binds to TNF with an affinity similar to that of TNFR (Schreiber *et al.*, 1996). Secreted SFV T2 also binds specifically to both TNFα and TNFß (Smith *et al.*, 1991). Each of these proteins may play a dual role during infection, acting as competitive anti-inflammatory molecules as well as inhibitors of TNF-induced apoptosis (Sedger and McFadden, 1996).

Disruption of MYX T2 attenuates virus replication in CD4+ rabbit T-lymphocytes, due to the onslaught of an apoptotic response (Macen *et al.*, 1996b). Surprisingly, the antiapoptotic activity of MYX T2 was found to be independent of TNF binding (Schreiber *et al.*, 1997). T2 deletion variants that could neither bind TNF, or be secreted into the extracellular medium blocked apoptosis as efficiently as the wild type protein indicating T2 may also act intracellularly, possibly as a dominant negative inhibitor of cellular TNF receptors (Schreiber *et al.*, 1997). Remarkably, CPV encodes three TNFR homologues (CrmB, CrmC and CrmD) that resemble the extracellular domains of TNFRs and compete effectively for TNF ligand binding (Hu *et al.*, 1994a; Loparev *et al.*, 1998; Smith *et al.*, 1996), suggesting that blocking TNF is important for a productive cowpox infection. However, the specific role of these CPV TNFR homologues in apoptosis modulation distinct from TNF binding remains to be experimentally determined.

Poxvirus antiapoptotic mechanisms are, however, not restricted to the suppression of cytokine or interferon induced apoptotic signals. MCV gene product MC066L is a novel selenoprotein with homology to glutathione peroxidase that acts as an antioxidant (Shisler *et al.*, 1998). MC066L has been found to protect human keratinocytes against apoptosis induced by UV irradiation and hydrogen peroxide and may play a role in long term virus

survival within the skin of infected patients (McFadden, 1998; Shisler *et al.*, 1998). A similar function for the recently described fowlpox FPV064 seems likely as FPV064 contains the glutathione peroxidase signature sequence including the active site for selenocysteine encoded by the opal codon (Afonso *et al.*, 2000).

Viral encoded apoptosis regulators are commonly associated with host range permissiveness. The host range proteins of several poxviruses, including the CPV CHOhr protein (CP77) (Spehner *et al.*, 1988), contain ankyrin-like repeats that provide interfaces for protein-protein interaction and are found in many cytosolic and cytoskeletal proteins. Introduction of CHOhr into VV allows replication and inhibits apoptosis in the normally nonpermissive Chinese hamster ovary cell line (Ink *et al.*, 1995; Spehner *et al.*, 1988). MYX virus encodes at least three virulence factors, M-T4 (Barry *et al.*, 1997), M11L (Opgenorth *et al.*, 1992), and M-T5 (Mossman *et al.*, 1996), which are associated with host range permissiveness and virulence. The MYX M-T4 gene encodes a novel protein, which is retained within the ER and is important for both the productive viral infection of lymphocytes *in vitro* and the classical features of lethal myxomatosis *in vivo* (Barry *et al.*, 1997). Deletion of MYX T4 results in an attenuated infection, characterized by significantly reduced numbers of secondary lesions in infected rabbits (Barry *et al.*, 1997). The inability of the virus to disseminate *in vivo* is likely due to inefficient viral suppression of apoptosis, as infection of rabbit CD4+ T -lymphocytes and peripheral blood lymphocytes with the MYX T-2 mutant virus results in the rapid induction of apoptosis (Barry *et al.*, 1997).

The MYX T4 gene sequence contains both an N-terminal signal sequence and a C-terminal RDEL ER retention sequence (Barry *et al.*, 1997). Deletion of the RDEL motif affects the stability of T4, but it does not affect localization of T4 to the ER indicating that other sequences are also involved (Hnatiuk *et al.*, 1999). The RDEL motif may fulfill additional functions, since infection of rabbit lymphocytes with the MYX T4 RDEL- mutant

virus results in an intermediate apoptosis phenotype compared with the wildtype and MYX T4 knockout mutant viruses (Hnatiuk *et al.*, 1999). Further, European rabbits infected with the recombinant MYX T4 RDEL- mutant virus exhibited an exacerbated edematous and inflammatory response at secondary sites of infections suggesting MYX T4 may have a dual or interrelated function in protecting infected lymphocytes from apoptosis and in modulating the inflammatory response to virus infection (Hnatiuk *et al.*, 1999).

The MYX M-T5 gene exhibits no significant sequence similarity to non-viral proteins (Mossman *et al.*, 1996), and disruption of M-T5 has no effect on the replication of MYX in rabbit fibroblasts. Infection, however, of rabbit CD4+ T-lymphocytes with this mutant M-T5 virus results in a rapid and complete shut down of both host and viral protein synthesis, which is accompanied by apoptosis induction (Mossman *et al.*, 1996). Within the European rabbit, disruption of M-T5 gives rise to a dramatic attenuation of the rapidly lethal MYX infection. Histological investigation suggests the attenuation of virulence observed with the M-T5 mutant virus results from the lack of progression of the infection past the primary site of inoculation, together with a rapid and effective inflammatory reaction (Mossman *et al.*, 1996).

M11L is a novel 166–amino acid membrane-associated MYX protein that exhibits little similarity with proteins outside the poxvirus family except for a putative transmembrane domain at its C-terminus (Graham *et al.*, 1992). M11L plays an important role in the virulence of MYX during host infection (Graham *et al.*, 1992; Opgenorth *et al.*, 1992). In contrast to MYX wild type infection, which gives rise to lethal symptoms of myxomatosis, targeted disruption of the M11L gene gave rise a highly attenuated, nonlethal disease in European rabbits, which was characterized by the formation of lesions with a profound proinflammatory response (Opgenorth *et al.*, 1992). M11L was subsequently identified as a virulence factor required to prevent apoptosis during MYX infection of rabbit

T-lymphocytes (Macen *et al.*, 1996b). Although M11L was originally defined as a cell surface associated protein, whose surface association was critical for the manifestation of viral virulence (Graham *et al.*, 1992), recent evidence indicates M11L is associated primarily with mitochondria both during infection and also independent of other viral proteins following eukaryotic expression (Everett *et al.*, 2000). M11L blocks staurosporine induced apoptosis independent of infection by preventing mitochondria from undergoing the apoptosis associated membrane permeability transition (Everett *et al.*, 2000). Consequently, M11L prevents caspase-3 activation and poly(ADP-ribose) polymerase cleavage suggesting that M11L acts directly at a step in an apoptotic cascade upstream of mitochondrial permeability transition and caspase-3 activation. This is the first direct evidence of a poxviral protein acting on mitochondrial induced apoptotic pathways. Indeed, mitochondrial targeting of M11L through a signal in its C-terminal 25 amino acids is essential for its anti-apoptotic function (Everett *et al.*, 2000). M11L is required to maintain the viability of primary rabbit monocytes/macrophages infected with MYX suggesting a key role for M11L in preventing infected macrophages from initiating a protective apoptotic response (Everett *et al.*, 2000). Thus, M11L likely acts as a virulence factor, promoting viral replication and spread through the inhibition of apoptosis.

Additionally, the recent sequencing of the fowlpox virus genome has led to the identification of the first reported poxviral member of the Bcl-2 gene family (Afonso *et al.*, 2000). FPV039 is a 143 amino acid protein that exhibits 29% identity over a 134 amino acid stretch with the Bcl-2 anti-apoptotic family member, BFL1. BFL1 is specifically expressed in bone marrow, spleen and thymus (Afonso *et al.*, 2000) suggesting a role for FPV039 in perhaps the modulation of apoptosis during fowlpox replication in cells of lymphoid origin. Although FPV039 contains both a BH1 and BH2 domain, it lacks additional BH3 and BH4 domains (Afonso *et al.*, 2000). It remains to be experimentally

determined whether FPV039 can modulate the cellular apoptotic response to infection, although from analysis of other viral bcl-2 homologues, this would seem highly probable.

The central theme evident from these studies is that the ability to suppress the host apoptotic response to infection is associated with viral host range permissiveness in tissue culture, replication capacity and virus virulence *in vivo*. The critical role of the EV RING finger protein p28 in promoting replication and virulence in mice, the natural host (Senkevich *et al.*, 1994), together with reports of RING finger proteins from viral and cellular origin, such as the baculovirus IAP protein (Crook *et al.*, 1993) and its mammalian homologs (Deveraux and Reed, 1999), in modulation of apoptotic responses pointed towards a possible role for the poxviral RING finger proteins in apoptosis regulation. Here, experimental evidence is presented, which indicates a novel role for SFV N1R and its EV homolog (p28), in apoptosis suppression. Overexpression of SFV-N1R in VV infected BGMK cells reduced virus induced apoptosis (Brick *et al.*, 1998). To clarify the role of N1R protein with respect to apoptosis and to examine whether the related EV p28 might also play a role in apoptosis protection, EV and the VV-N1R virus were tested for their ability to interfere with apoptosis induced by different signals. Cells infected with either VV or EV were protected against apoptosis induced by these agents.

In comparison to VV wildtype infection, overexpression of the SFV N1R protein protected VV infected HeLa cells from apoptosis induced by UV light, but not from apoptosis induced by TNF ligand or anti-Fas antibody. Disruption of the EVp28 gene sensitized EV infected HeLa cells to apoptosis induced by UV light, but did not affect EV sensitivity to Fas and TNF induced apoptosis, indicating EVp28 specifically blocks UV induced apoptotic signaling pathways.

Immunoblot analysis indicated EVp28 blocks processing of procaspase-3 suggesting EVp28 acts upstream of this protease in response to UV induced apoptotic

signals. Further, evidence is presented which indicates that the requirement of EVp28 to promote replication and virulence *in vivo* may be related to apoptosis suppression. The number of progeny virus harvested from p28- mutant EV virus infected cells compared to wild type EV was similar following mock UV induced apoptosis, but significantly reduced following apoptosis induction by UV.

MATERIALS AND METHODS

Sources of viruses, cells, tissue culture reagents, oligonucleotides, restriction-modification enzymes and protocols for virus growth, plasmid preparation, protein analysis, immunological analysis, *E. coli* transformation and eukaryotic cell transfections have been described in Chapter one.

For transient eukaryotic expression of SFV N1R and poxviral orthologs in HeLa cells under the human cytomegalovirus enhancer/promoter in plasmids pCI-neo (Brondyk, 1995) (Promega, Madison, WI, USA), pCMVβ (MacGregor and Caskey, 1989) (Clontech Laboratories, Inc., Palo Alto, CA, USA), pBK-CMV (Alting-Mees *et al.*, 1992) (Stratagene, La Jolla, CA, USA) and pRc/CMV (Kung *et al.*, 1991) (Invitrogen, San Diego, CA) QIAGEN purified plasmids were transfected into HeLa cells. For isolation and selection of stable pBK-CMV and pRc/CMV transfectants with the aminoglycoside G-418 (Geneticin®; GibcoBRL) the DNA-lipofectin mixture was removed 12 hours post transfection and replaced with complete D-MEM. 24 hours later, cells were detached from the dishes with SSC containing trypsin, washed in PBS, and one fifteenth of the original cell population seeded back into 6 well dishes. 6 hours later, the medium was replaced with complete D-MEM containing 250, 500, 750 or 1000 μg/ml G418 (stock prepared in 100 mM HEPES, pH 7.3). Four days later, and every four days thereafter until termination of the experiment, the medium was removed, cells washed briefly with PBS and fresh complete D-MEM and G418 added back to the cells. Cells were harvested at day 8 and day 16 with SSC and trypsin. Two fifths of the cells were used for western blot analysis of poxviral RING finger expression using Mab H119 (see below), one-fifth to expand the cell population under selective conditions and the remainder was used to prepare stocks that were stored at -80°C.

Recombinant DNA

Oligonucleotide primers are shown in Table 3. In order to ascertain if SFV N1R and related homologs from MYX and VV could substitute for EVp28 in promoting virulence within the context of EV infection, an EVp28 gene replacement recombination vector, pT7/gpt-gus-p-p28ko was obtained from Dr. R. Mark .L Buller (Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center, St. Louis, Missouri, USA). This plasmid construct is based upon pE/L-gg (Cao and Upton, 1997). It contains a gpt-gus selectable marker and a cassette inserted into the *Nde* I and *Hind* III sites of pE/L-gg, which consists of left and right EV genome EVp28 gene flanking regions surrounding a cloning site containing *Not* I and *Nhe* I sites. In order to clone the mAb tagged gene sequences for N1R and its poxviral orthologs from pMSN1 based vectors into this recombination vector, primers EVZ-N and EVZ-C were designed to create a *Nhe* I site upstream of the initiating methionine of the mAb epitope and a *Not* I site downstream of the *Bam*H I site in the multiple cloning region of pMSN1, respectively. Because of cloning site limitations this introduced a Leu residue after the initiating Met residue of the wild type epitope tag. Gene sequences were amplified by PCR (denaturation at 94°C for 2 mins, 6 cycles of denaturation at 92°C for 30 sec, annealing at 48°C for 1 min, extension at 72°C for 2 mins, followed by 25 cycles of 92°C for 30 sec, 72°C for 2 mins 30 sec with a final extension at 72°C for 3 mins and cooling to 4°C). Individual PCR reactions (Total 50 µl) contained 5 µl of 10x reaction buffer (200 mM Tris-HCl [pH 8.8], 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton-X-100, 1000 µg/ml nuclease-free BSA), 0.4 µl dNTPs (25 mM each dNTP), 125 ng each of primers EVZ-N and EVZ-C, 2.5 U cloned *Pfu* DNA polymerase and 50 ng of plasmids pMSN1 containing the mAb tagged gene sequences for N1R or MAB tagged gene sequences for the EV, IHDW and WR orthologs.

PCR products were isolated Gel purified PCR products were digested with *Nhe* I and *Not* I, repurified from agarose and ligated into similarly digested pT7/gpt-gus-p-p28ko. Ligations, carried out at 16°C overnight, contained 100 ng vector, 50 ng gel purified insert, 4

µl 5X buffer [250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v PEG-8000)], 1.5 µl 10 mM ATP and 1 µl (1U/ µl) recombinant T4 DNA ligase in a total volume of 20 µl. Ligations were transformed into competent *E. coli* DH5α and recombinant clones were identified by PCR using the PCR program ENZ and 1 µl of presumptive recombinant overnight LB culture as template. PCR positive recombinants were used to prepare plasmid DNA for restriction digestion. Positive recombinants were verified by restriction enzyme digestion with *Nhe* I and *Not* I.

For transfection of EVp28 replacement constructs into VV infected BGMK cells, plasmid midipreps were purified on QIAGEN tip 100 columns as described in chapter one. QIAGEN purified constructs were transfected into VV infected cells (MOI=10) and transient expression examined by western blot analysis using mAb H1119.

To restore both the normal wild type EVp28 gene promoter sequence and MAB epitope sequence of pMSN1 in EVp28 gene replacement constructs, site directed mutagenesis was employed using the QuikChange™ (Braman *et al.*, 1996) Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Primers EVR2A and its complement EVR2B were designed to restore the wild type promoter sequence of EVp28 and delete the gene sequences encoding the additional Leu residue in the mAb epitope. These primers consequently destroyed the *Nhe* I site in these gene replacement vectors and thus facilitated screening of mutant plasmids.

PCR reactions (50 µl total) contained 5 µl of 10x reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.8), 20 mM $MgSO_4$, 1% Triton-X-100, 1 mg/ml nuclease-free BSA), 10 ng respective pT7/gpt-gus-p-p28ko gene replacements, 125 ng each of designed mutagenic primer EVR2A and its complement EVR2B, 1 µl of dNTP mix, double distilled water (ddH20) to 49 µl and 1 µl of Pfu DNA polymerase (2.5 U). Reactions were overlaid with 30 µl of mineral oil. PCR was carried out using the MUTG PCR program (denaturation at 95°C for 30 sec followed by 18 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min and extension at 68°C for 16 mins. Reactions

were cooled to 4°C prior to addition of 1 µl of restriction enzyme *Dpn* I (10U/µl) to each amplification reaction, gentle mixing by pipetting, microcentrifugation for 1 minute at 14, 000 rpm and incubation at 37°C for 1 hour 30 mins (to digest the parental methylated DNA). 1 µl of the *Dpn* I-treated DNA was added to 50 µl Epicurian Coli® XL1-Blue supercompetent cells Stratagene, La Jolla, CA, USA), mixed by gently swirling and placed on ice.

30 mins later, the transformation reactions were heat-pulsed for 45 seconds in a 42°C water path and immediately placed on ice for 2 mins. 0.5 ml of preheated 42°C NZY+ broth (1% casein hydrolysate, 0.5% yeast extract, 0.5% NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 20 mM glucose) was added to each transformation mixture and incubated at 37°C for 1 hour with shaking at 235 rpm. 250 µl of each transformation mixture was plated on LB agar plates containing 50 µg/ml ampicillin. Plates were allowed to dry by sitting on the bench for 30 mins prior to inversion in a 37°C incubator for 16-20 hours. Mutagenic plasmids were identified by release of the *Nco* I-*Bam*H I gene fragment and the absence of a *Nhe* I site compared to the parental template

For eukaryotic expression of SFV N1R and poxviral orthologs independent of viral infection, four eukaryotic expression vectors driven by the human cytomegalovirus (CMV) immediate early gene promoter were employed. pCI-neo (5474 bp; Neo^R, Promega) (Brondyk, 1995), contains both a *Sal* I and a *Sma* I site within its multiple cloning site (MCS). As such this facilitated subcloning of N1R mAb from pMSN1 (Upton *et al.*, 1994). pMSN1 was digested with *Bam*H I, gel purified and then blunt ended at 37°C for 1 hour with Klenow DNA polymerase. Reactions (50 µl) contained 10 µl of 5x Klenow buffer (50 mM Tris-HCl, 25 mM $MgCl_2$, 37.5 mM DTT), 1.0 µg gel purified *Bam*H I digested pMSN1, 20 µM of each dNTP and 1 µl (5 U) of recombinant DNA polymerase I large fragment (Klenow). Following the addition of EDTA to 10 mM, the reaction was heated to 95°C for 2 mins to denature the polymerase, and the DNA recovered from

agarose. Following digestion with *Sal* I, the released approximately 750 bp fragment was recovered from agarose and ligated into pCI-neo that had been digested with *Sal* I and *Sma* I. Following transformation into *E. coli* DH5α and selection on LB and Kanamycin , recombinants were identified by PCR and release of the N1R MAB tagged ORF by digestion with *Sal* I and *Not* 1.

pBK-CMV (4518 bp; Neo^R, Stratagene) (Alting-Mees *et al.*, 1992) contains *Sal* I and *Bam*H I restriction enzyme sites within its MCS. The MAB tagged ORFs for SFV N1R and tagged orthologs from MYX, EV and IHDW were subcloned from pMSN1 into pBK-CMV by digestion with *Sal* I and *Bam*H I and ligation into similarly digested pBK-CMV. Following selection on LB containing 50 μg/ml kanamycin, recombinant clones were identified by the presence of an additional *Nco* 1 site and release of the *Sal* I-*Bam*H I MAB-ORF cassette.

The mAb tagged ORFs for N1R and MYX, EV and IHDW were additionally subcloned from pBK-CMV into pRc/CMV (5.5 kb; Amp^R Neo^R; Invitrogen) (Kung *et al.*, 1991). Following *Sal* I digestion of pBK-CMV vectors harbouring the *Sal* I-*Bam*H I N1R gene cassettes and blunt ending with recombinant DNA polymerase I large fragment (Klenow), the approximately 750 bp mAb tagged ORF gene fragments were released by digestion with *Apa* I. The gene inserts were ligated into pRc/CMV that had been digested with *Hin*d III, blunt ended and subsequently digested with *Apa* I. Recombinant clones were identified by PCR using MAB-N primer in combination with SN2-C for SFV and MYX N1R ORFs, primers VN2-N and VN2-C for EV and VV orthologs and digestion with *Nco* I and *Eag* I.

pCMVβ vector (7.2 kb Amp^R; Clontech) (MacGregor and Caskey, 1989) is a mammalian reporter vector designed to express beta-galactosidase in mammalian cells from the human cytomegalovirus immediate early gene promoter. The beta-galactosidase gene can be excised using the *Not* I sites at each end to allow other genes to be inserted into the vector backbone for expression. Primers MAB-NODE1 was designed to place a *Not* I site

flanking the mAb tagged N1R ORFs from SFV, MYX, VV-WR, VV-IHDW and EV in pMSN1 . Following PCR (Program ENZ) using 2.5 U Pfu polymerase (Stratagene) and primers MAB-NODE1 and EV-C (contains *Not* I site), PCR products were digested with *Not* I, and ligated into similarly digested pCMVβ. Following transformation into *E. coli* DH5α and selection on LB agar containing 50 μg/ml ampicillin, recombinant clones containing the gene sequences in the correct orientation were identified by digestion with *Nde* I and *Hin*d III or alternatively *Bam*H I.

Epitope tagging of EV-p28		
VN2-N	5' CCCCATGGAATTCGATCCTGCC	(creates *Nco* I site)
VN2-C	5' AAGGATCCTTAGTTAACTAGCTTATAGAA	(creates *Bam*H I site)
Replacement of EVp28 with SFV, MYX and VV orthologs		
EVZ-N	5' CCCCAAAAGCTAGCGACTGACATTGATATGCAT	(creates *Nhe* I site)
EVZ-C	5' CCAAAAAAGCGGCCGCCTAGCGGGCCCGGATCC	(creates *Not* I site)
To restore wildtype EVp28 promoter sequence by site directed mutagenesis		
EVR2A	5' CACTGTTTAGTCGCGGATATGGCGACTGACATTGATATGC	(destroys *Nhe* I site)
EVR2B	5' GCATATCAATGTCAGTCGCCATATCCGCGACTAAACAGTG	(destroys *Nhe* I site)
Eukaryotic expression cloning		
MAB-NODE1	5' GGCATATAAAGCGGCCGCATATGGCGACTGACATTGATATGC	(creates *Not* I site)
EVZ-C	5' CCAAAAAAGCGGCCGCCTAGCGGGCCCGGATCC	(creates *Not* I site)

Table 3. List of oligonucleotide primers utilized in cloning procedures (chapter 2)

Apoptosis

Internucleosomal DNA cleavage (DNA laddering) analysis following agarose gel electrophoresis of isolated cellular DNA.

A variety of isolation methods for analysis of apoptotic DNA by agarose gel electrophoresis were employed (Gong *et al.*, 1994; Herrmann *et al.*, 1994; Martin *et al.*, 1990), however, the method presented here (Koyama and Miwa, 1997) resulted in the strongest resolution of apoptotic DNA ladders following ethidium bromide staining. For the DNA fragmentation assays, samples of 10^6 BGMK cells grown to 80% confluence were used. Virus infections used a MOI of 3. Adherent and floating cells were harvested and pooled at various times. Cells were detached from 6 well dishes with SSC supplemented with trypsin and pelleted at 240 g for 5 mins. Cell pellets were gently resuspended in 100 µl complete D-MEM and transferred to eppendorf tubes. Cell suspensions were lysed by the addition of 400 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA) containing 0.6% SDS. 125 µl of 5 M NaCl was added to the cell lysates and mixed gently by tube inversion 6 times. Following overnight incubation at 4°C, samples was centrifuged in the cold room at 14, 000 g for 45 mins and 200 µl of the supernatants transferred to eppendorf tubes containing 5 µl of RNase A (10 mg/ml; Sigma Chemical, St. Louis, MO, USA). Following incubation at RT for 5 mins, 5 µl of Proteinase K (20 mg/ml; Sigma Chemical) was added and samples incubated at 55°C for 1 hour. 0.8 mls of 100% ethanol was then added, mixed by gentle inversion, and tubes were incubated overnight at -80°C. Precipitated DNA was recovered by centrifugation at 14, 000 g for 45 mins in the cold-room. DNA pellets were washed once with 0.8 ml cold 70% ethanol and then dried under vacuum (Vacufuge RC 10.10; Canberra Packard Canada, Mississauga, Ontario, Canada). DNA was solubilized by the addition of 10 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA) and incubated at 4°C for 5 hours prior to the addition of 10 µl of 6 x DNA loading

buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% sucrose, 50 mM EDTA). Samples (10 µl) were loaded per well into a 1.5% agarose gel and electrophoresis carried out at 2V/cm for 12 hours. *Hind* III digested lambda phage DNA was utilized as size markers. Gels were stained with 0.5 µg/ml ethidium bromide in ddH_20 for 10 mins prior to visualization by a transilluminator (302 nm; UV Transilluminator TM-36, UVP-Ultraviolet Products, San Gabriel, CA) and photography.

ELISA detection of cytoplasmic apoptotic nucleosomes

For ELISA quantification of apoptosis, a sandwich assay was performed using a pair of mAbs specific for two nucleosomal epitopes to capture and detect cytoplasmic nucleosomes (Salgame *et al.*, 1997). The hybridoma cell lines LG11-2 and PL2-3 were generously provided by Dr. Marc Monestier (Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania, USA). mAbs were prepared by Immuno-Precise Antibodies Ltd. (Victoria, BC). The capture mAb, LG11-2, an IgG2aκ obtained from an autoimmune MRL/*lpr* mouse, is specific for the N-terminus of histone H2B. The detection mAb, PL2-3, is an IgG2aκ specific for the nucleosome subparticle composed of histones H2A, H2B and DNA. To prepare cytoplasmic lysates for ELISA analysis, 10^6 cells were harvested (670 g for 5 mins), washed with 2 mls PBS, pelleted by centrifugation and resuspended in 50 µl of ice cold lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl pH 7.5). Following incubation on ice for 30 mins, cellular debris and nuclei were pelleted by centrifugation of the lysate at 425 g for 5 mins. 30 µl of the cytoplasmic supernatant was carefully transferred to Eppendorf tubes and stored at -20°C.

Microtitre plates (Falcon ref.3912) were coated with 50 µl well of LG11-2 capture mAb (2 µg/ml) diluted (1:200) in 0.05 M carbonate buffer (0.159% Na_2CO_3, 0.293% $NaHCO_3$, pH 9. 6). After overnight incubation at 4°C or 2 hours incubation at RT, plates were washed 3 times with PBS-Tween (0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4,

0.02% KCl, 0.05% Tween-20, pH 7.4) and blocked with 250 µl of PBTN (PBS-Tween containing 0.02% sodium azide and either 1% BSA [Fluka Biochemika] for ELISA with BGMK cell lysates or 1% normal goat serum [Immunoprecise Antibodies Ltd.] for HeLa cell lysates) for 1 hour at RT. Following 3 washes for 5 minutes each with PBS-Tween, 50 µl of cytoplasmic lysate diluted in PBS-Tween containing 0.02% sodium azide was added to each well and incubated overnight at 4°C. Plates were then washed 4 times for 5 mins each with PBS-Tween and incubated with 50 µl/well of biotinylated PL2-3 detection antibody diluted (1: 2000) in PBTN for 1.5 hours at RT. After three washes with PBS-Tween for 5 mins each, 50 µl well of streptavidin alkaline-phosphatase conjugate (Amersham Life Science, Arlington heights, IL, USA) diluted in PBTN was added. After 30 minutes incubation at RT, plates were washed for 3 times for 5 mins each in PBS-Tween and 3 times in diethanolamine buffer (10 mM diethanolamine [Sigma Chemical], 0.02% sodium azide, 0.5 mM $MgCl_2.6H_20$ [pH 9.8]). p-nitrophenyl phosphate tablets were dissolved in RT diethanolamine buffer to a final concentration of 1 mg/ml, prior to addition of 150 µl/well. Plates were developed in the dark at RT for 30-60 minutes prior to measuring optical densities at 405 nm using an ELISA plate reader (Automated EIA plated reader EL310; Bio-Tek Instruments, Inc., Burlington, Vermont, USA).

For ELISA analysis of VV-induced apoptosis of BGMK cells, VV infections were performed as described for the DNA fragmentation assays and cycloheximide (CHX; Sigma, Chemical) was used at 200 µg/ml. The ELISA was performed using 2.5 X 10^4 cell equivalents per well. For evaluation of VV protection from apoptotic signal induction by UV and CHX, samples of 10^6 BGMK cells, at 80% confluence, were either mock infected; infected with VV (MOI=3) or infected with VV-N1R (MOI=3). At 12 hours post infection, mock infected and virus infected cells were either mock treated or treated with 200 µg/ml CHX or exposed to UV light exposure for 10 min (302 nm; UV Transilluminator TM-36, UVP-Ultraviolet Products) from underneath the dish surface. Cells were harvested at 12 hours post treatment and the ELISA performed using 2.5 X 10^4 cell equivalents per well.

In order to determine the optimal concentrations of Anti-Fas (Anti-human Fas [CD95], murine monoclonal CH-11; Kamiya Biomedical Company, Seattle, WA, USA) or TNF (human, recombinant [*E. coli*] tumor necrosis factor-α; Boehringer Mannheim, Indianapolis, IN, USA) in combination with CHX for ELISA detection of apoptosis induction in HeLa cells, samples of 10^6 HeLa cells, at 80% confluence were treated with varying concentrations of Anti-Fas (50 ng/ml, 500 ng/ml, 1250 ng/ml), TNF (10 ng/ml, 20 ng/ml, 50 ng/ml) either alone or in combination with CHX (1 μg/ml, 15 μg/ml, 40 μg/ml). Cells were harvested at 12 hours post treatment and the ELISA performed using 2.5 X 10^4 cell equivalents per well.

For evaluation of VV and EV protection from apoptotic signal induction by Anti-Fas, TNF and UV exposure, samples of semi-confluent HeLa cells (6 x 10^5) in six well dishes were infected with virus at MOI=3. At twelve hours post-infection, cells were either mock treated, or treated with TNFα (10 ng/ml; Boehringer Mannheim) and CHX (15 μg/ml; Sigma), anti-Fas (50 ng/ml; CH-11 antibody, Kamiya Biomedical) plus CHX (15 μg/ml), CHX (15 μg/ml) alone, or a two minute exposure to UV light (302 nm; UV Transilluminator TM-36, UVP-Ultraviolet Products) from underneath the dish surface. Twelve hours later adherent and floating cells were harvested and processed for the ELISA assay. The ELISA was performed using 3.75 X 10^3 cell equivalents per well.

For titration of the effects of varying UV exposure times on apoptosis protection by EV samples of semi-confluent HeLa cells (6 x 10^5) in six well dishes were infected with EV or the p28-mutant EV at MOI=3. At 12 hours post-infection, infected cells were either mock exposed to UV or exposed for 0.5, 1, 2, 5 or 7.5 mins. Infected cells were harvested 12 hours later and the ELISA performed using 3.75 X 10^3 cell equivalents per well.

DAPI detection of apoptotic nuclei.

For 4',6'-diamidino-2-phenylindole (DAPI) analysis of nuclear morphology, HeLa cells were grown in 8 chamber slides (SuperCell, Fisher Scientific, Pittsburgh, PA) or, for UV analysis, on coverslips in six well dishes. Cells were either mock infected or infected with virus (MOI=3). At twelve hours post-infection, cells were either mock treated, or treated with TNFα (10 ng/ml; Boehringer Mannheim) and CHX (15 μg/ml; Sigma), anti-Fas (50 ng/ml; CH-11 antibody, Kamiya Biomedical) plus CHX (15 μg/ml), CHX (15 μg/ml) alone, or a two minute exposure to UV light (302 nm; UV Transilluminator TM-36, UVP-Ultraviolet Products) from underneath the dish surface. Twelve hours later, treated and mock-treated samples were fixed with ice cold 70% ethanol for 15 mins (Otto, 1994). Cells were then rinsed in PBS and stained with 500 ng/ml DAPI (Sigma) in PBS for 5 mins in the dark. After rinsing in PBS, coverslips were mounted, viewed and photographed with epifluorescence using excitation and emission filters of 365 and 420 nm respectively.

Flow cytometric analysis using propidium iodide (PI) staining

Samples of semi-confluent HeLa cells (2 x 10^6) in six well dishes were either mock infected or infected with EV or p28-mutant EV (MOI=5). At ten hours post-infection, cells were mock exposed or exposed to two minutes of UV light from underneath the dish. Fourteen hours later, adherent and floating cells were harvested and pelleted through 2 mls of complete D-MEM by centrifugation at 167 g for 10 mins (Beckman GS-15). Cells were washed in 5 mls of ice cold PBS and resuspended in 2 mls of modified "saline GM" (PBS containing 5% glucose) (Crissman and Hirons, 1994). Following the addition of 150 μl of 80% glycerol to the cell suspension, cells were permeabilized by the dropwise addition of 95% ice cold ethanol to a final concentration of 80% ethanol, mixed by gently inversion 2 times and incubated at 4°C for 15 mins. Thereafter, cells were stored at -20°C until analysis was performed. Prior to analysis, cells were harvested by centrifugation as above, washed

with 5 mls of PBS and resuspended in 2 mls of PBS. 50 µl of 50 µg/ml PI (Sigma) plus 2 mg/ml RNase A (Sigma) in PBS was then added and samples incubated in the dark for 15 mins at RT and thereafter kept in the dark at 4°C until FACS analysis was performed (Nicoletti *et al.*, 1991). The DNA content of 10, 000 cells per sample was measured using a FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA) equipped with an argon-ion laser (488 nm) and the data were registered on a logarithmic scale. The light scatter characteristics were simultaneously measured and all data were acquired and analyzed using CELLQuest software (Becton Dickinson).

Biochemical assessment of procaspase-3 activation

Samples of semi-confluent HeLa cells (6 x 10^5) in six well dishes were infected with viruses at MOI=3. At eight hours post-infection, cells were either mock exposed, or exposed to UV light (302 nm) for two minutes. Sixteen hours later, adherent and floating cells were harvested. Rabbit polyclonal IgG anti human CPP32 (H-277) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) recognizes the full length precursor form of human CPP32 (procaspase-3). Protein samples for detection of CPP32 were separated on 18% SDS-PAGE, transferred to PVDF membrane and blocked with 5% Hipure liquid gelatin (Norland Products, New Brunswick, NJ, USA) in PBS containing 0.1% Tween-20 (Aldrich Chemical, Milwaukee, WI, USA) for 90 minutes at RT (Lee *et al.*, 1994). Blots were incubated with polyclonal CPP32 (H-277) (1: 200) in PBS containing 1% BSA (Fluka Biochemika) and 0.1% Tween-20 overnight at 4°C. Bound antibody was detected by horseradish peroxidase conjugated goat anti-rabbit IgG (1:5000) (Caltag Laboratories inc., Burlingame, CA, USA) and Supersignal® chemiluminescent substrate (Pierce, Rockford, IL, USA) as described by the manufacturer.

Effects of UV induced apoptosis on viral titer

Semi-confluent HeLa cells (6×10^5) in six well dishes were infected with EV or p28-mutant EV (MOI=3). At four hours post-infection, infected cells were mock exposed or exposed to UV light for either one or two minutes from underneath the dish surface. 48 hours later, cells were harvested, resuspended in 0.5 mls of ice cold hypotonic swelling buffer (10 mM Tris [pH 8.0], 2 mM $MgCl_2$) and following incubation on ice for 15 min, virus released by three successive rounds of freeze-thaw. Virus samples were sonicated (Branson Sonifier 450; Branson Ultrasonics, Danbury, CT, USA) at 50% cycle, an output of 9, for 1 min, prior to the addition of 0.5 ml 2X complete D-MEM, and serial dilution for titer determination.

Viral titrations were performed using monolayers of BGMK cells in 12 well plates. Infections were performed using a 0.2 ml infection volume. After incubation for 3 days, the medium was removed and virus plaques stained with 1 ml of 1% v/v crystal violet (BDH Chemicals, Toronto, ON, Canada) made in NBF. After 10 mins incubation, at room temperature (RT), the stain was removed, plates inverted on paper towels and allowed to dry. Plaques were counted from the underneath of the dish using a felt marker, and plaque counts within the range of 20-200 utilized to estimate virus titer. The percent reduction in EV progeny virus titer after UV exposure was expressed as a percentage of the unexposed virus infected samples by dividing the respective EV titer following UV exposure for 1 or 2 mins by the titer of the respective EV mock UV exposed samples.

Statistical analysis

Results from the apoptotic ELISA, DAPI staining and viral titer experiments were analyzed with the Students t test using the statistical package in Microsoft Excel (Microsoft Corp.) and are presented as mean and either standard error of the mean (SEM) or standard deviation (SD)

RESULTS

Expression of SFV N1R protein delays apoptosis in VV infected cells

Apoptosis, often referred to as programmed cell death, is a physiological process in which the cell actively participates in a cascade of biomolecular events that result in the demise and disposal of the cell. It is important in development, in the regulation of cell numbers, and as a defense mechanism to remove unwanted and potentially dangerous cells, such as self reactive lymphocytes, tumor cells and virus infected cells (Nagata, 1997). We decided to test the hypothesis that the SFV N1R protein might be involved in blocking virus-induced apoptosis because a number of cellular proteins with RING motifs have key roles in regulation of apoptosis (Deveraux *et al.*, 1997; Hu *et al.*, 1994b; Lee and Choi, 1997; Rothe *et al.*, 1994; Thut *et al.*, 1997; Uren *et al.*, 1996) and inactivation of the EV ortholog (p28) significantly reduces virus virulence (Senkevich *et al.*, 1994). Apoptosis was measured using a standard assay for the fragmentation of host chromosomal DNA into small oligonucleosomes (Koyama and Miwa, 1997). Apoptosis of cells infected with wild type VV and a VV that overexpresses the SFV N1R protein (VV-N1R) was compared. No DNA laddering was seen at 24 hr, but VV infected BGMK cells showed significant laddering by 48 hr. In contrast, cells infected with VV-N1R showed little or no laddering at this time or at 54 hr (Figure 20).

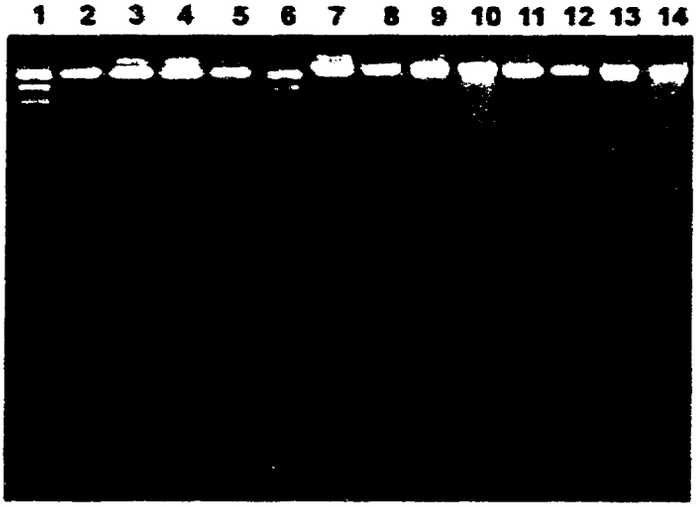


Figure 20. Protection from apoptosis by expression of SFV N1R in VV infected BGMK cells. Ethidium bromide stained agarose gel. Lanes: 1 and 6, HindIII digested lambda DNA; lanes 2, 7 and 11, mock infected; lanes 3, 8 and 12, mock infected, no serum; lanes 4, 9 and 13, VV-N1R infected; lanes 5, 10 and 14, VV infected. Samples taken at 24 hr, lanes 2-5; 48 hr, lanes 7-10; 54 hr, lanes 11-14.

In similar assays, it was also observed that EV infected cells did not produce apoptotic DNA ladders even at 54 hr post infection (data not shown) suggesting that EV encodes gene products which act to suppress the hosts apoptotic response to infection. The sensitivity of this laddering assay (Eastman, 1995; Gavrieli *et al.*, 1992; Salgame *et al.*, 1997) is such that the majority of infected cells are likely undergoing apoptosis and contributing to the observed signal. This is supported by visual inspection of cells in the following experiment that used an ELISA to measure cytoplasmic apoptotic nucleosomes (Figure 21).

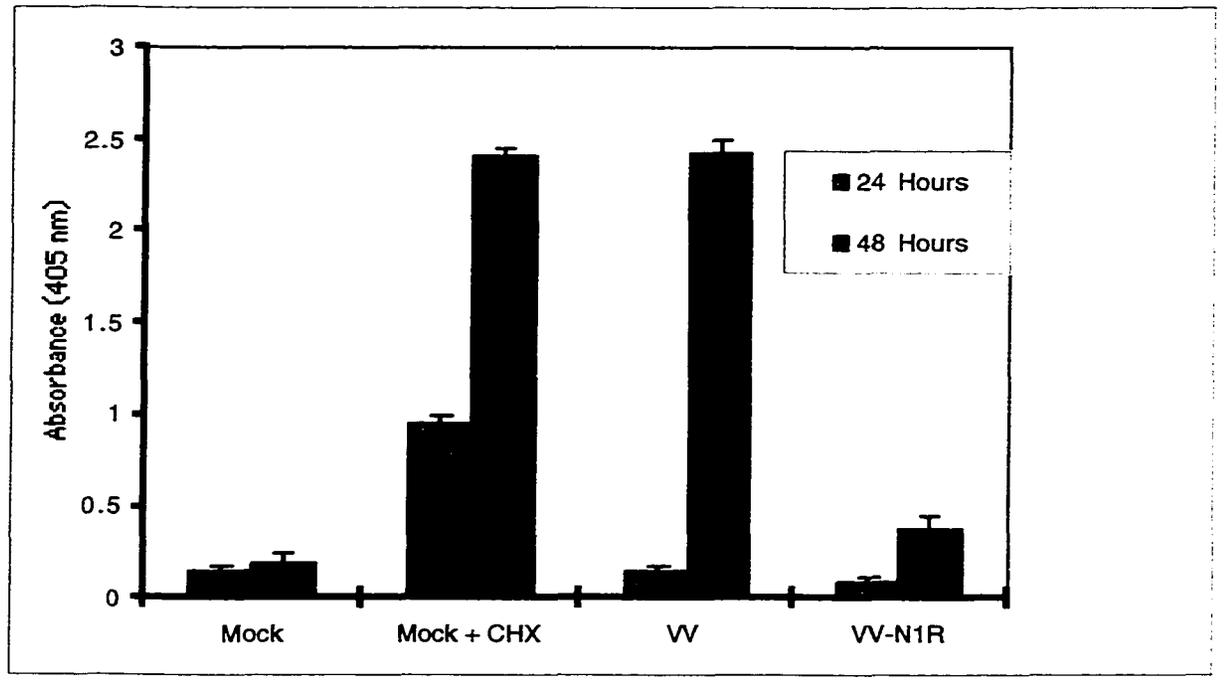


Figure 21. Protection from apoptosis by expression of SFV N1R in VV infected BGMK cells. ELISA detection of cytoplasmic oligonucleosomes.

The assay, which is at least 10-fold more sensitive than the laddering assay, shows a similar absence of any apoptosis at 24 hr and inhibition of nucleosome release by VV-N1R at 48 hr (Figure 21). Apoptosis was induced in control uninfected cultures by treatment with CHX (200 μg/ml), microscopic inspection of these cells indicated that all cells had undergone morphological changes. A lower CHX concentration (30 μg/ml) has previously been shown to induce apoptosis in more than 60% of HeLa cells (Kettle *et al.*,

1997). The extent of nucleosome release in the VV infected cells at 48 hr was comparable to that observed in a CHX-induced apoptotic culture (Fig. 21). Thus, the expression of SFV N1R protein in VV infected cells significantly reduced virus-induced apoptosis.

In order to confirm that protection from apoptosis by SFV-N1R was not due to a difference in replication between the two viruses, BGMK cells were infected with VV or VV-N1R and virus yield at time points up to 36 hours post infection was determined using a standard plaque assay (Figure 22).

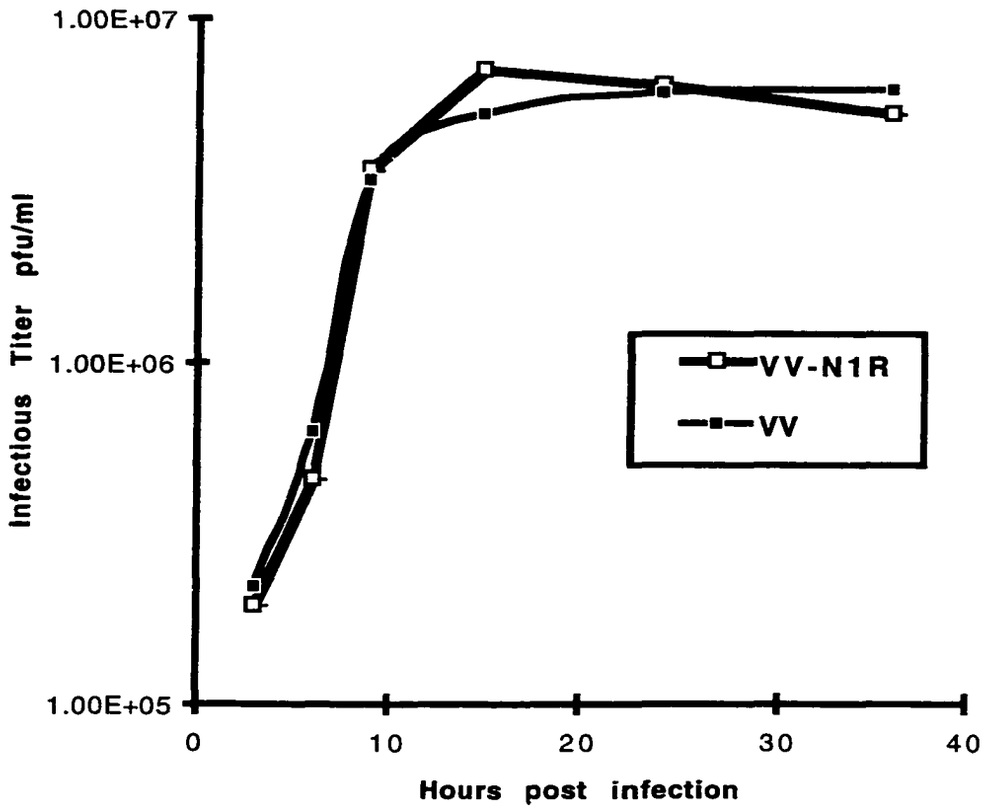


Figure 22. Single step growth curves of wild type VV (strain IHDW) and recombinant VV-N1R viruses following infection of BGMK cells. Duplicate samples of BGMK cells in 6-well dishes were infected with VV or VV-N1R at MOI=3 and parallel plates were harvested at 3, 6, 9, 15, 24 and 36 hours post infection. Titers of progeny virus were determined in triplicate using a standard plaque assay.

Consistent with previous studies that found no difference in replication between EV-WT and the p28$^-$ mutant EV virus following infection of a variety of cell culture lines (Senkevich *et al.*, 1994; Senkevich *et al.*, 1995), overexpression of SFV N1R in VV-infected cells was not found to affect virus replication at least for up to 36 hours post infection.

Expression of SFV N1R protein protects VV infected BGMK cells from UV induced apoptosis.

In order to determine if N1R could protect from apoptosis induction by different agents, such as UV or CHX, BGMK cells were either mock infected; infected with VV (MOI=3) or infected with VV-N1R (MOI=3). At 12 hours post infection, mock infected and virus infected cells were either mock treated , or treated with 200 μg/ml CHX or a 10 mins UV light exposure. Treatment of mock infected BGMK cells with either UV light or CHX resulted in apoptosis induction, whereas little apoptosis is detectable following mock treatments of both mock infected and virus infected samples at 24 hours post-infection (Figure 23). In contrast, VV infected cells, afforded protection from UV and CHX induced apoptosis. Greater protection from UV induced apoptosis was afforded by VV-SFV-N1R compared to VV suggesting a role for the poxviral RING finger proteins in protection from UV light induced apoptosis. No appreciable difference, however, in the levels of detectable cytoplasmic oligonucleosomes was found between VV and VV-SFV N1R following CHX treatment.

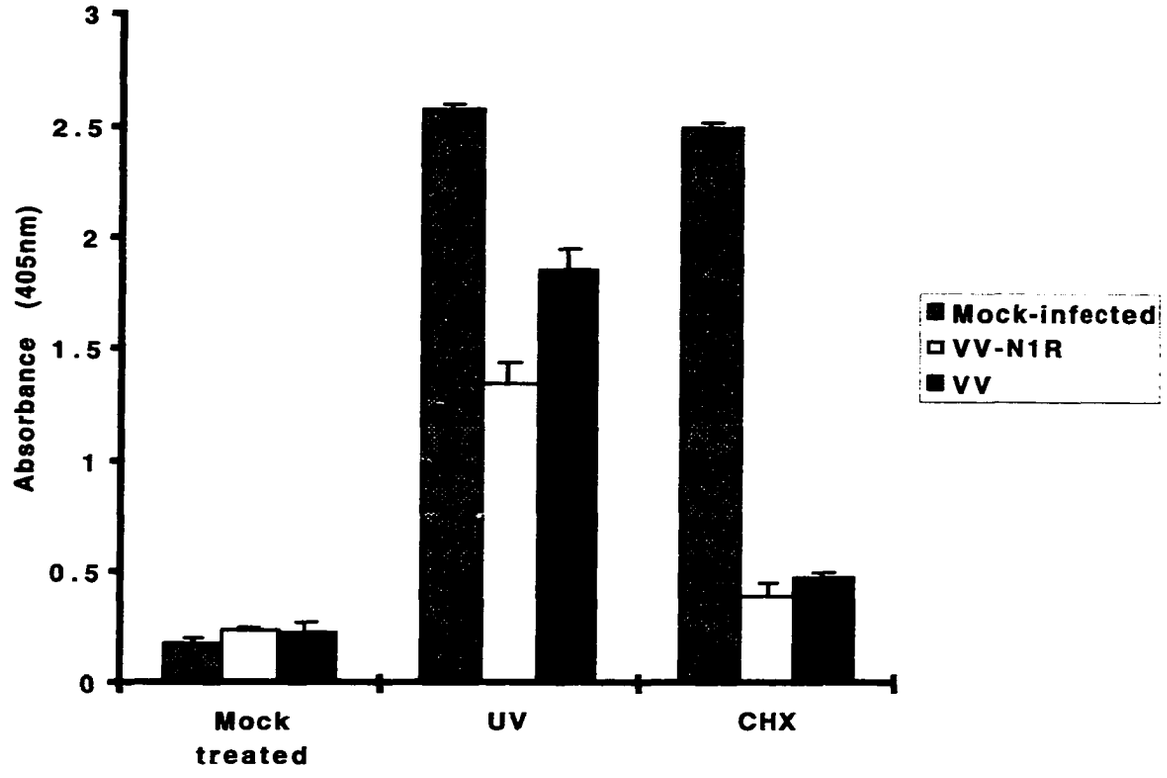


Figure 23. ELISA detection of apoptosis following UV or CHX treatment of VV infected BGMK cells. BGMK cells were mock infected or infected at MOI=3 with VV or VV-N1R. The ELISA used 2.5 x 10^4 cell equivalents per well. Duplicate samples were collected and each was assayed in triplicate.

Two well characterized inducers of apoptosis are the cytokines FasL and TNF (Cleveland and Ihle, 1995). Because recombinant human TNFα and anti-human Fas antibody were commercially available, the ELISA assay system was also set up to analyze HeLa cell lysates. In order to determine the optimal concentration of these reagents for ELISA detection of apoptosis, HeLa cells were treated with varying concentrations of Anti-Fas or TNF either alone or in combination with CHX (Table 4).

	Anti-Fas (50 ng/ml)	Anti-Fas (500 ng/ml)	Anti-Fas (1250 ng/ml)	TNF (10 ng/ml)	TNF (20 ng/ml	TNF (50 ng/ml	Untreated
No CHX	0.9	1.9	1.3	0.6	0.6	0.4	0.3
CHX (1 µg/ml)	2.8	2.4	2.3	1.5	1.4	2.1	0.6
CHX (15 µg/ml)	2.8	2.3	2.3	2.3	2.3	2.3	0.6
CHX (40 µg/ml)	2.8	2.5	1.7	2.1	2.4	2.0	0.6

Table 4. ELISA titration of apoptosis induction following treatment of HeLa cells with varying concentrations of anti-Fas Ab or TNF alone or anti-Fas Ab or TNF with CHX. Untreated samples serve as negative controls for backround levels of apoptosis. Samples were analyzed 12 hours post-treatment. The ELISA was performed using 2.5 x 10^4 cell equivalents per well. Numerical values represent the mean absorbance readings at 405 nm.

Consistent with the survival associated induction effects of TNF, optimal death induction by this cytokine required coadministration of CHX. Interestingly, the addition of CHX was also found to enhance the apoptosis inducing effects of anti-Fas antibody treatment. In the following experiments, CHX, TNF and anti-Fas were used at 15 µg/ml, 10 ng/ml and 50 ng/ml, respectively.

SFV N1R protein and EV-P28 protect from UV, but not from TNF or anti-Fas induced apoptosis of HeLa cells.

Because of the critical role of EVp28 in the pathogenicity of this virus, it was of interest to determine if the EVp28 RING homolog could also protect from apoptosis. Apoptosis was measured in mock infected HeLa cells and cells infected with either VV,

VV-N1R, EV-WT or p28$^-$ mutant EV virus following treatment with either TNF + CHX, anti-Fas + CHX or UV exposure. CHX alone treated, and untreated samples serve as controls.

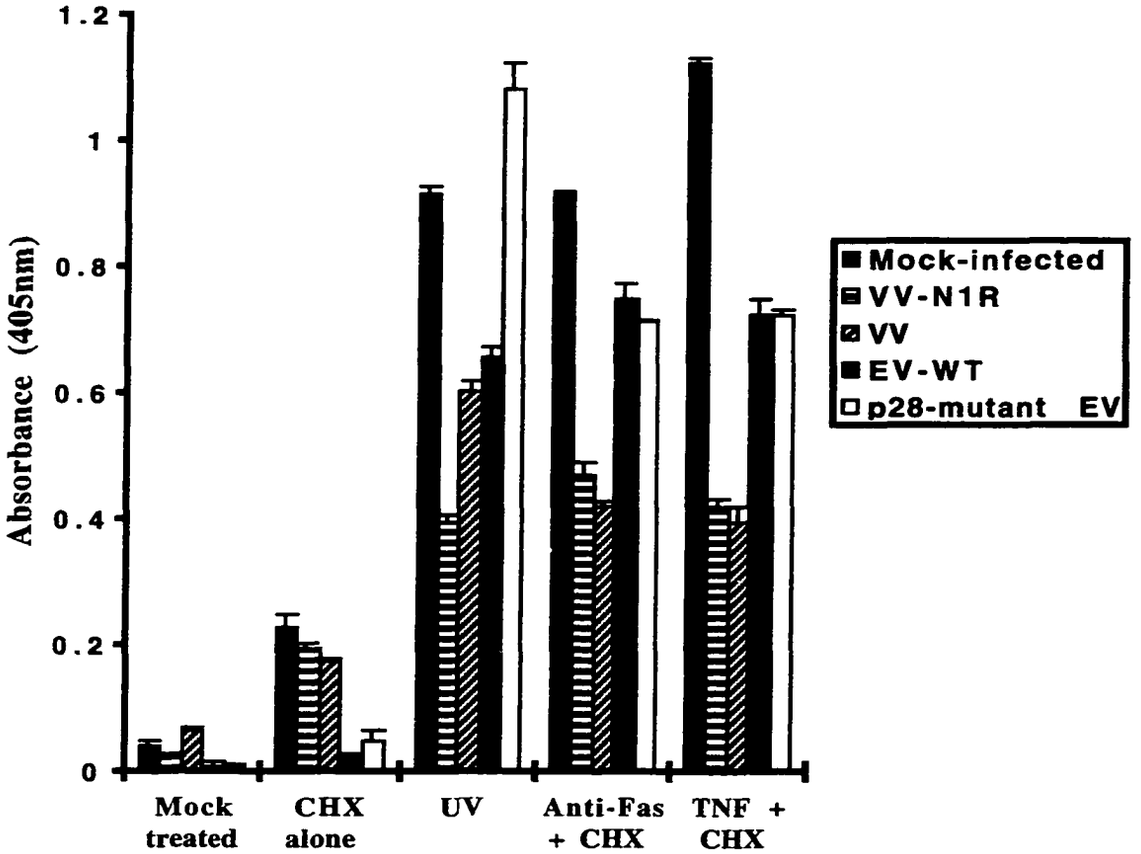


Figure 24. ELISA detection of apoptosis following Anti-Fas, TNF or UV induced apoptosis of VV and EV infected HeLa cells. HeLa cells were mock infected or infected at 3 pfu per cell with VV, VV-N1R, EV-WT or the p28$^-$ mutant EV virus. Mock infected or infected cells were either untreated; UV irradiated; treated with CHX + anti-Fas Ab; treated with CHX + TNF or with CHX alone at 12 hours post infection. Duplicate samples were collected at 24 hours and each was assayed in triplicate.

Treatment with UV light, TNF or anti-Fas induced apoptosis in mock infected and virus infected cells, whereas no apoptosis is detectable following mock treatment of similar cells 24 hours post-infection (Figure 24). VV and EV-WT infection protected cells from TNF, anti-Fas and UV induced apoptosis. Protection from UV induced apoptosis was repeatedly afforded by VV-SFV-N1R compared to VV (Student *t* test, $P = 0.007$) and to a greater extent by EV-WT compared to the $p28^-$ mutant EV virus ($P = 0.002$), suggesting a role for the poxviral RING finger proteins in protection from UV light induced apoptosis. No appreciable difference, however, in the levels cytoplasmic oligonucleosomes was found either between EV and the $p28^-$ mutant EV virus, or between VV and VV-SFV N1R following Fas or TNF treatments suggesting that the apoptosis suppressing activity of the poxviral RING finger proteins is restricted to blocking UV induced signals. Thus, overexpression of the SFV-N1R RING finger protein increases the ability of VV to protect from UV induced apoptosis and disruption of the EVp28 RING finger gene sensitizes EV infected cells to undergo UV induced apoptosis.

Although VV infection has previously been shown in various reports to protect from TNF and anti-Fas induced apoptosis (Dobbelstein and Shenk, 1996; Heinkelein *et al.*, 1996; Kettle *et al.*, 1997), this is the first indication that VV infection protects from UV light induced apoptosis. With respect to EV-WT infection, to the best of our knowledge, this is the first study to examine the response of EV-WT infection to such death inducers. Although EV encodes a *crmA* homolog, recent evidence suggests that the perforin mediated apoptotic pathways may be more significant in controlling EV infections *in vivo* (Mullbacher *et al.*, 1999). Interestingly, EV-WT infection has less a protective effect against such death inducers compared to VV (Figure 24). This, however, may be reflective

of lower viral anti-apoptotic protein expression levels by EV-WT due to the slower growth of EV-WT compared to VV in tissue culture.

In order to further examine the protective role of EVp28 in response to UV induced irradiation, apoptosis was measured by ELISA in HeLa cells infected with either EV-WT or the $p28^-$ mutant EV virus following increasing time of UV exposure. Although no difference in the levels of apoptosis is seen in the absence of UV exposure, apoptosis levels increase rapidly following UV treatments of the $p28^-$ mutant EV virus compared to EV-WT (Figure 25).

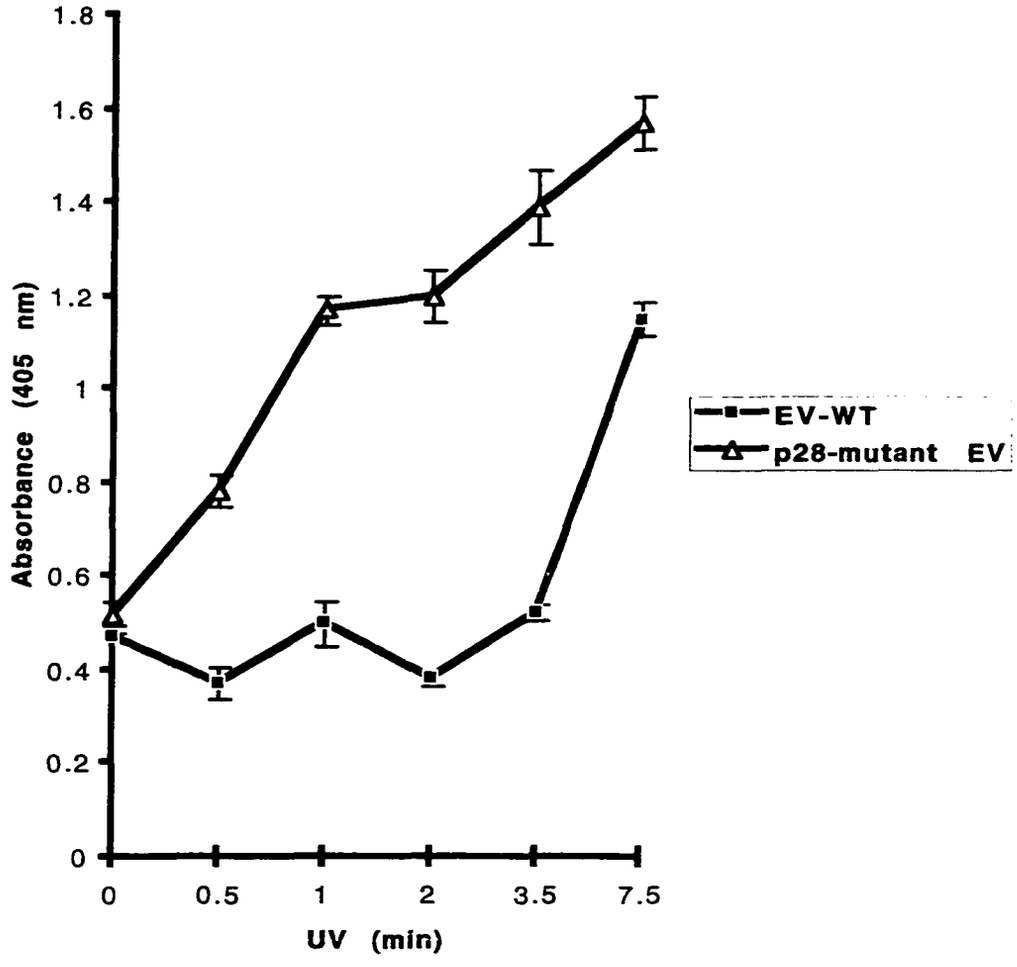


Figure 25. ELISA detection of apoptosis following increasing UV exposure of EV infected HeLa cells. Duplicate samples were collected and each was assayed in duplicate.

At short UV exposure times of EV-WT infected HeLa cells, the level of apoptotic oligonucleosomes remains similar to that of unexposed virus infected samples. However, prolonged UV treatment of EV-WT infected cells results in apoptosis induction, which is likely due to saturation of the UV apoptosis protective EVp28 function in response to overwhelming UV proapoptotic signals within the infected cell. Apoptosis was also independently assessed by examination of nuclear morphology using the DNA fluorochrome DAPI. Apoptotic cells exhibit increased fluorescence with this dye permitting them to be distinguished from normal cells. DAPI staining analysis (performed blind) supported the ELISA based findings (Figure 26A).

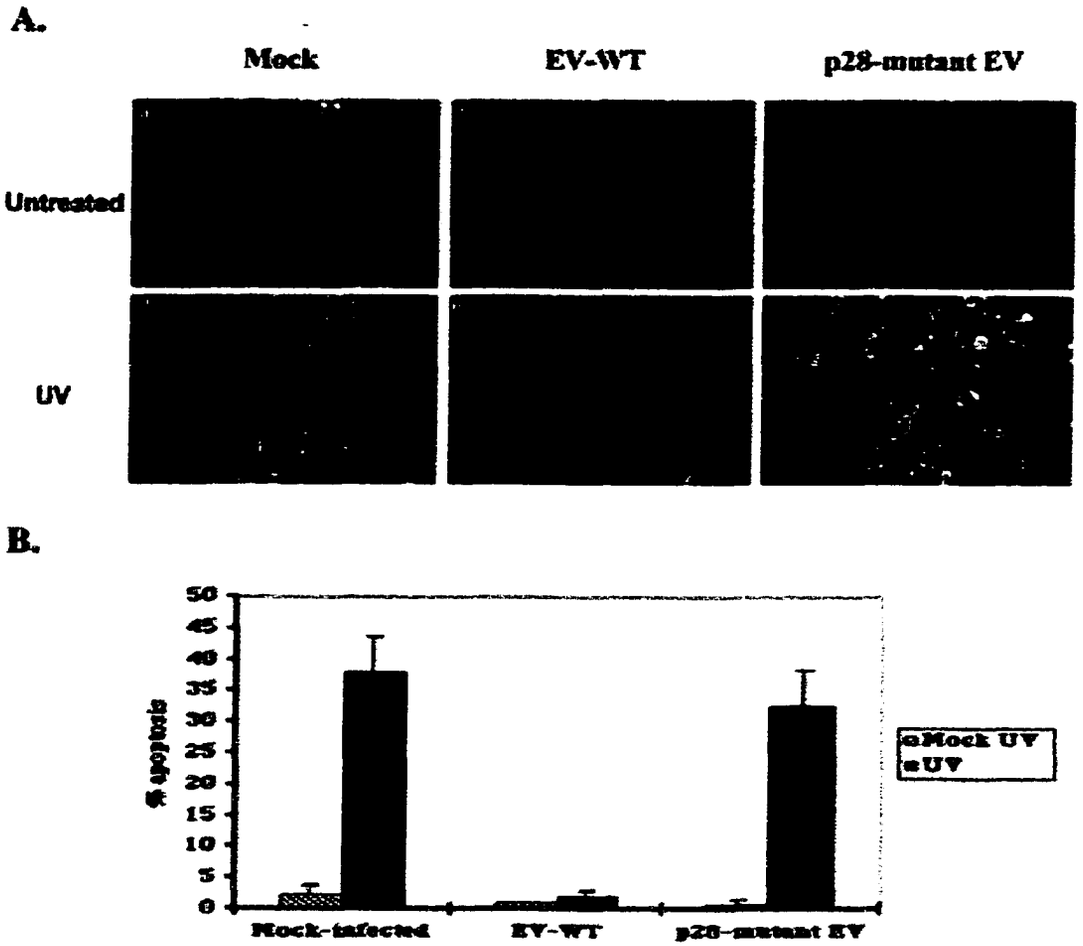


Figure 26. DAPI analysis of nuclear morphology. A. HeLa cells grown on coverslips in six well dishes were mock infected or infected at MOI=3 with EV-WT or the p28$^-$ mutant EV virus. Mock infected or infected cells were either untreated (a, b and c) or UV irradiated (d, e and f) at 12 hours post infection. 12 hours later cells were fixed and stained with DAPI to visualize nuclei. Examples of cells with apoptotic morphology are indicated by the arrowhead (in panel f). B. Average percentage of apoptotic cells from five randomly chosen fields, with SEM.

Treatment with TNF + CHX or anti-Fas + CHX induced apoptosis in approximately 80% of mock infected cells, whereas both the EV-WT and p28$^-$ mutant EV virus infected cells exhibited approximately 20% apoptosis (data not shown). Although no appreciable difference between EV-WT and the p28$^-$ mutant EV virus in response to these reagents was observed, UV treatment of the p28$^-$ mutant EV virus infected HeLa cells (Figure 26A panel f) showed nuclear condensation, nuclear blebbing and the presence of apoptotic bodies (indicative of late stages of apoptosis) that were also present in mock infected UV treated HeLa cells (Figure 26A panel d). In contrast, UV treatment of EV-WT infected cells showed no signs of apoptosis induction (Figure 26A panel e); nuclear morphology was similar to that observed in both untreated mock infected and virus infected cells. The average number of apoptotic cells from five randomly chosen fields was determined (Figure 26B). Again, EV-WT was found to strongly inhibit UV induced apoptosis ($P = 0.003$) compared to the p28$^-$ mutant EV virus.

DAPI staining, however, relies on analysis of adherent cells. To measure apoptosis in all cells, flow cytometric analysis (FCA) of propidium iodide (PI) stained cells was employed. The fragmented DNA of apoptotic cells incorporates less PI than the intact DNA of healthy cells, thus the DNA content as determined on a single cell level by FCA after DNA staining allows a more accurate quantification of the degree of apoptotic (hypodiploid) cells in a population (Nicoletti *et al.*, 1991) (Figure 27). Cells showing decreased fluorescence after PI staining (marked by a gate in Figure 27) appear to the left of those in G1 or G2 phase of the cell cycle. Mock infected or virus infected HeLa cells show no difference in the level of apoptosis at 24 hours post infection, however, UV treatment results in elevated levels of apoptosis in mock infected and p28$^-$ mutant EV virus infected cells (Figure 27d and 27f), compared with UV treated EV-WT infected cells (Figure 27e).

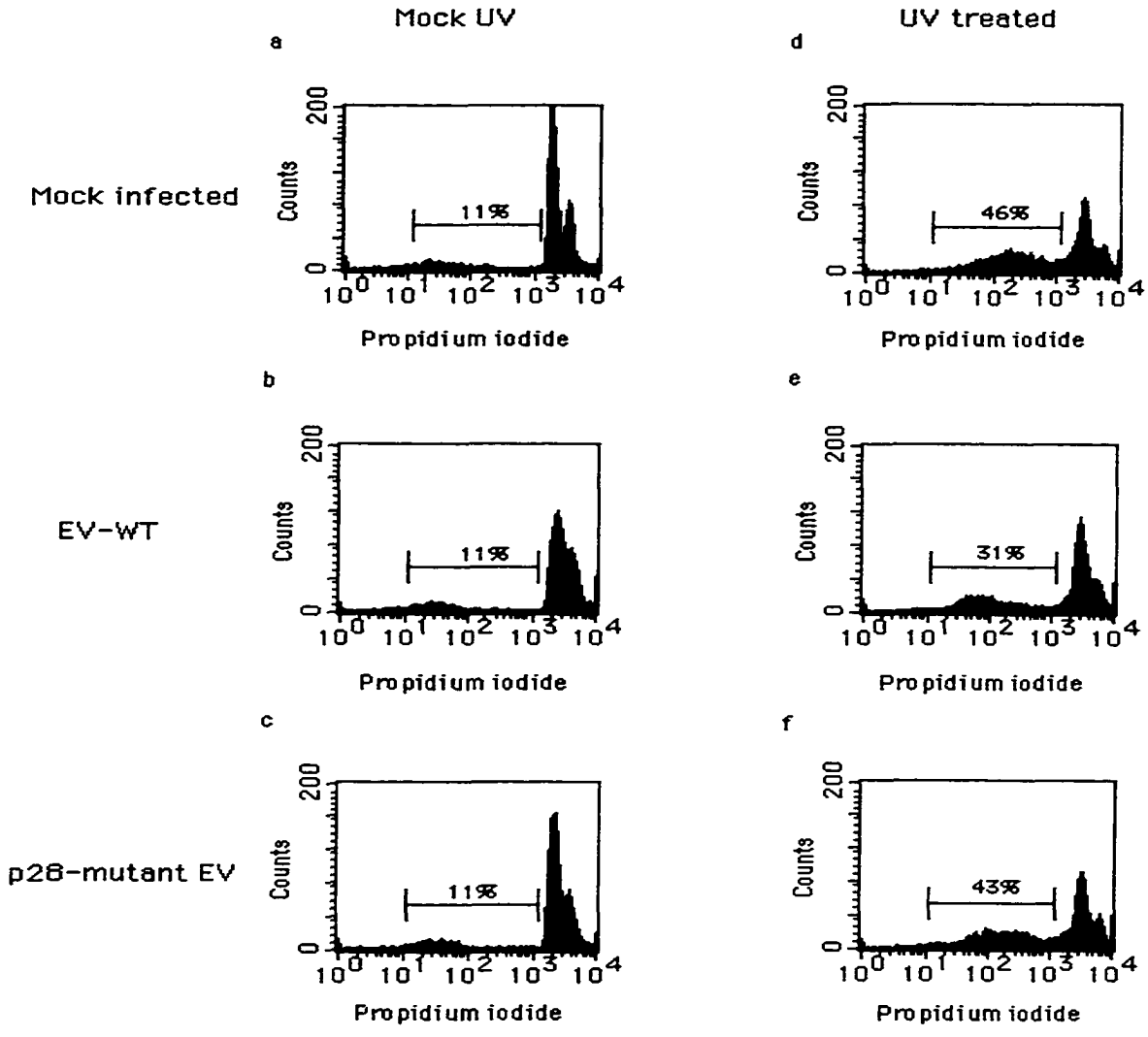


Figure 27. FCA analysis of the DNA content of mock infected or virus infected cells. HeLa cells were mock infected or infected at 5 pfu per cell with EV-WT or the p28$^-$ mutant EV virus. Cells were either mock UV irradiated or UV irradiated for 2 mins at 10 hours post infection. Cells were harvested at 24 hours post infection, permeabilized and stained with PI. Cell counts are plotted against PI fluorescence; the percentage of cells of the hypo-diploid (apoptotic) population is indicated in each panel.

Apoptotic cells undergo condensation and shrinkage which results in altered light scatter characteristics. The scatter profiles of these cell populations were measured simultaneously by FCA (Figure 28).

The scatter plots of untreated mock infected and virus infected cells show similar profiles of background levels of apoptosis at 24 hours post infection (Figure 28a-c), but UV treatment of mock infected and $p28^-$ mutant EV virus infected cells (Figure 28d and 28f) resulted in a shift towards decreased cell size that was accompanied by decreased PI fluorescence. Again, consistent with a role for the EVp28 RING finger protein in UV induced apoptosis suppression, UV treatment of EV-WT infected HeLa cells inhibited the shift in cell size and PI fluorescence towards the apoptotic region (Figure 28e) compared to both UV treated mock infected and $p28^-$ mutant EV virus infected cells. Thus FCA analysis supports the ELISA and DAPI based findings which indicate that although EV-WT infection protects infected cells from UV induced apoptosis, the EVp28 gene product has a specific involvement in UV induced apoptosis suppression.

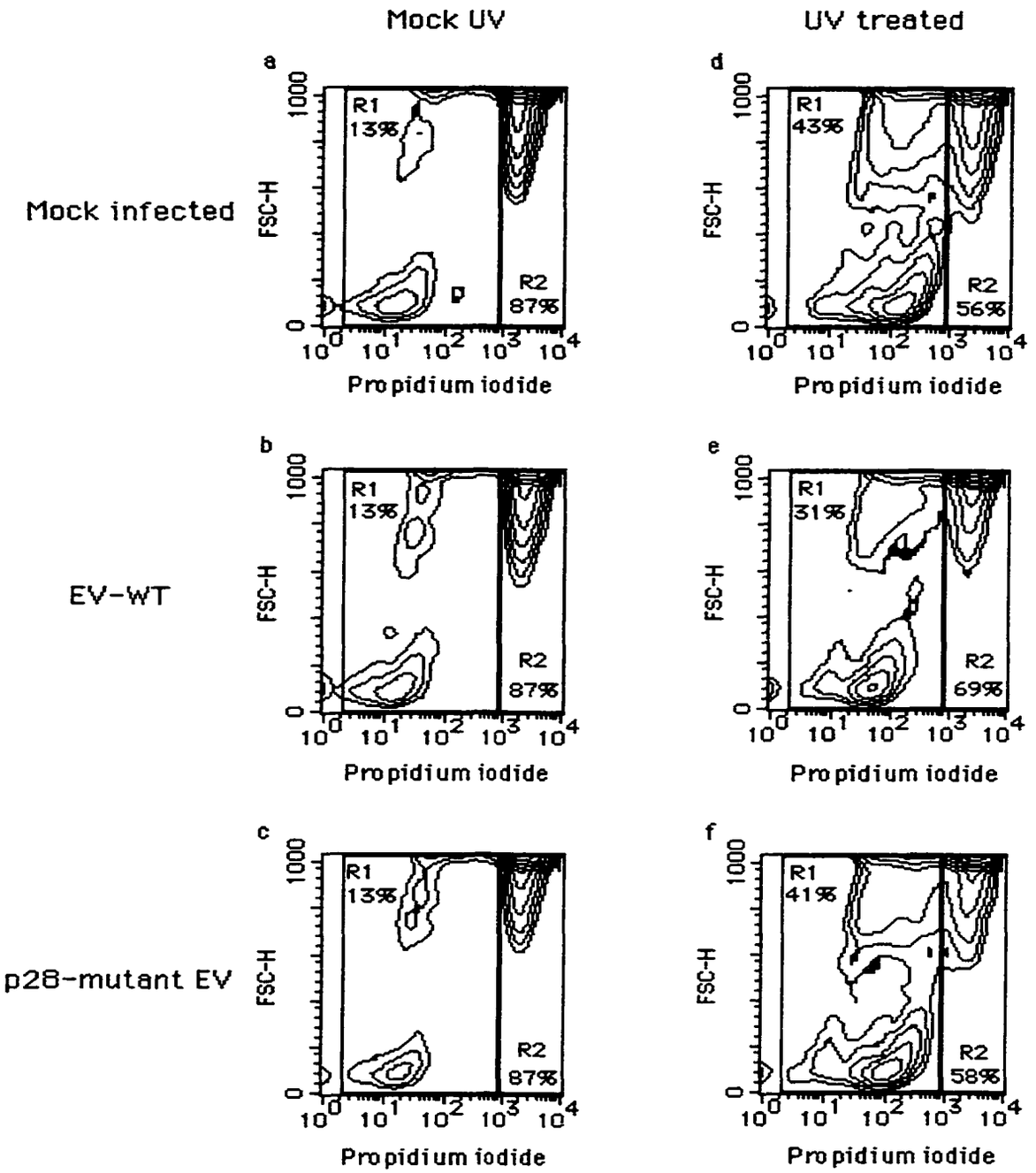


Figure 28. FCA analysis of the light scatter characteristics of mock infected or virus infected cells. Forward scatter (FSC; cell size) is plotted against PI fluorescence and the percentage of cells in the apoptotic (R1) and normal (R2) regions is indicated.

EV-P28 acts upstream of caspase-3 in response to UV irradiation

Caspase-3 is central to the execution of apoptosis in response to several stimuli (Janicke *et al.*, 1998; Porter and Janicke, 1999; Schlegel *et al.*, 1996; Woo *et al.*, 1998). The enzyme is synthesized in cells as an inactive 32 kDa precursor which is proteolytically cleaved into the 17 kDa and 12 kDa subunits of the mature caspase-3 during apoptosis. Several of the cellular targets of activated caspase-3 have been identified. These include important DNA repair enzymes such as poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase and the recently identified inhibitor of caspase-activated deoxyribonuclease (ICAD) (Enari *et al.*, 1998; Han *et al.*, 1996; Sakahira *et al.*, 1998; Tewari *et al.*, 1995b). Inhibition of caspase-3 has been shown to block apoptosis (Nicholson *et al.*, 1995) and the biological importance of this protease is demonstrated by the phenotype of caspase-3 knockout mice, which do not undergo normal apoptosis in certain tissues and die shortly after birth (Kuida *et al.*, 1996). An antibody that detects full length human caspase-3 was used to examine caspase-3 cleavage after UV irradiation of infected cells. The 32 kDa proform of caspase-3 is present in similar amounts in untreated mock infected, EV-WT and $p28^-$ mutant EV virus infected samples at 24 hours post infection. UV treatment, however, results in greatly diminished levels of procaspase-3 in both mock infected and $p28^-$ mutant EV virus infected cells (Figure 29).

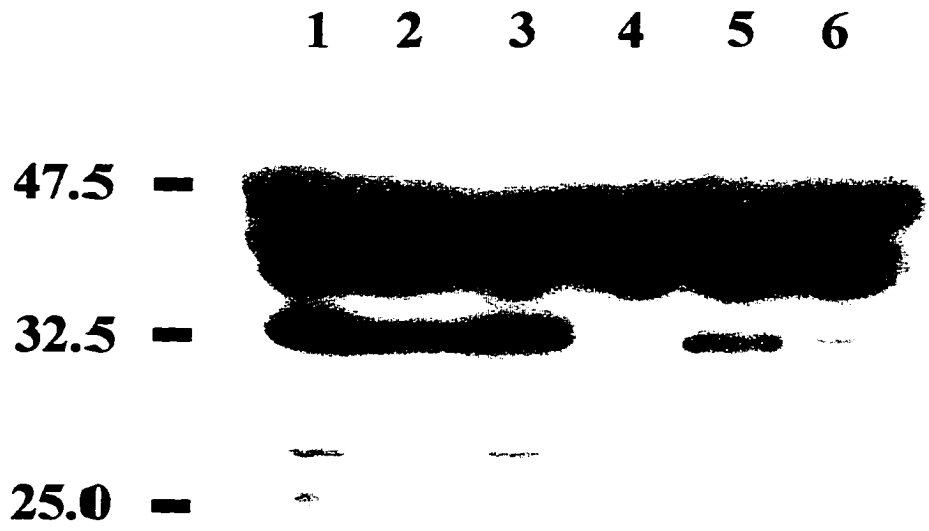


Figure 29. Western blot analysis of procaspase-3 (CPP32) in extracts from mock infected or virus infected HeLa cells following mock UV or UV treatment. Cells were either treated with UV (2 min exposure) or mock UV treated at eight hours post infection. Lanes: 1 & 4) Mock infected; 2 & 5) EV-WT infected; 3 & 6) p28$^-$ mutant EV virus infected. Lanes 1, 2 and 3 mock treated; Lanes 4, 5 and 6 UV treated. Standard proteins (kDa) are denoted with bars.

Correlating with the apoptotic assay results of inhibition of UV induced apoptosis by EVp28, procaspase-3 was consistently protected from cleavage in lysates prepared from UV irradiated EV-WT infected cells compared to UV irradiated p28$^-$ mutant EV infected and mock infected irradiated cells (Figure 29). This indicates that p28 acts upstream of caspase-3 activation in response to UV irradiation. Although the immunoblot did not detect either of the activated subunits of caspase-3 following UV irradiation, there were greatly diminished levels of proform caspase-3 in the p28$^-$ mutant EV virus infected UV treated cells. It is unlikely that this is due to different rates of procaspase-3 synthesis and degradation in virus

infected cells because the levels of procaspase-3 are similar between mock infected and EV virus infected samples in the untreated cells. The cross-reacting proteins provide a useful control to confirm sample loading equivalency between lanes. The reduced presence of procaspase-3 in both UV irradiated mock infected and p28$^-$ mutant EV infected cells is wholly consistent both with our previously observed role of EV-p28 in protection against UV-induced apoptosis and the essential nature of procaspase-3 activation to promote the nuclear and morphological changes of apoptosis (Frutos *et al.*, 1999; Kimura *et al.*, 1998; Miller *et al.*, 1998; Nemoto *et al.*, 1998).

In order to test whether SFV N1R and homologs from EV and VV could protect from apoptosis independent of viral infection, the epitope tagged poxviral ORFs were cloned using a variety of methods into four eukaryotic expression vectors driven by the human cytomegalovirus (CMV) immediate early gene promoter. Following transient expression in HeLa cells or selection for neomycin resistant clones with the antibiotic G418, expression of SFV N1R and the poxviral orthologs could not be detected by western blot analysis using mAb H1119. In retrospect, particularly with evidence of RING finger proteins acting as E3 ubiquitin ligases and indeed subject to possible ubiquitination transfer reactions, these transient expression experiments should be repeated in the presence of proteosome inhibitors. Another reason that these poxvirus genes were not expressed from the CMV promoter is that they may contain cryptic slice signals that cause the mRNA to be spliced when transcribed in the nucleus rather than the cytoplasm as it is normally in poxvirus infections.

Restricted Replication of the p28$^-$ mutant EV virus following UV exposure

A central question in beginning this research study was how expression of EVp28 promoted virulence with the natural host, the mouse. The finding of a role for EVp28 and SFV N1R in apoptosis protection provides a plausible mechanism through which these viral factors promote viral fitness. Indeed, the critical role of host cell apoptosis as an antiviral

defense mechanism, restricting viral replication and titer, is supported by the growing number of viral encoded genes that function to suppress apoptosis. In order to ascertain if SFV N1R and related homologs from MYX, VV could substitute for EVp28 in promoting virulence within the context of EV infection, an EVp28 gene replacement recombination vector, pT7/gpt-gus-p-p28ko was obtained from Dr. R. Mark .L Buller (Department of Molecular Microbiology and Immunology, Saint Louis University Health Sciences Center, St. Louis, Missouri, USA). Epitope tagged gene sequences for N1R and its poxviral orthologs were cloned into pT7/gpt-gus-p-p28ko and following transfection into VV infected cells, N1R expression was analyzed by western blot analysis using mAb H1119. Because of restriction site limitations within this vector, the EVp28 wild type promoter region upstream of the RNA start site -2 AT -1 was deleted and a codon coding for a Leucine residue inserted after the initiating methionine of the epitope tag.
Following transient expression in VV infected cells, N1R and its orthologs were undetectable by western blot analysis (data not shown). As such, site directed mutagenesis was employed to restore the wild type initiator sequence and epitope tag. However, following transfection into VV infected cells, protein expression was again undetectable by western blot analysis and the research project terminated. In hindsight, these transfections should be repeated with EV infected cells, since the initiator promoter and core regions of EVp28 do not closely match those of VV (Moss, 1996a), possibly leading to a failure in mRNA expression.

Previous studies suggested that p28 was critical for the replication of EV in murine resident peritoneal macrophages based on the result that no viral factories were detected by Hoechst dye staining in most of the $p28^-$ mutant EV virus infected macrophages compared with EV-WT infection (Senkevich *et al.*, 1995). In order to further examine a correlation between suppression of UV-induced apoptosis by EVp28 and virus replication capability, HeLa cells were infected with either EV-WT or the $p28^-$ mutant EV virus, treated with UV light for 0, 1 or 2 mins at 4 hours post-infection. At 48 hours post-infection, virus was

harvested and titred. UV irradiation reduced the number of progeny virus recovered from the p28$^-$ mutant EV virus infected cells compared to EV-WT infected cells (Table 5).

	Log $_{10}$ of virus titres ± SD[a]		*% unexposed virus*	
UV exposure (mins)	**EV-WT**	**p28-mutant EV**	**EV-WT**	**p28-mutant EV**
0	6.95 ± 0.36	6.94 ± 0.35[b]	100.00	100.00
1	6.03 ± 0.34	5.85 ± 0.33[c]	11.93	8.58
2	5.23 ± 0.34	4.55 ± 0.31[d]	1.85	0.38

Table 5. Reduction in progeny viral titer following increasing exposure to UV. HeLa cells were infected with virus and UV irradiated at 4 hours post infection. Virus was harvested at 48 hours post infection and titrated on BGMK cells. [a]The arithmetic mean values of two independent experiments are indicated with standard deviations (SD). [b]Student's t test indicate no significant difference from the value for EV-WT following mock UV treatment (t = 0.19, P = 0.43). [c, d]Significantly different from the value for EV-WT by the student *t* test at 1 mins UV exposure (t = 3.8, P = 0.03) and at 2 mins UV exposure (t = 28.2, P = 0.0006). Progeny virus yield following UV exposure is expressed as a percentage of the unexposed virus infected sample.

There is approximately a 1.4 fold difference in titer of the p28$^-$ mutant EV compared to EV-WT following one minute of exposure (P = 0.03) that is increased to a 4.9 fold difference following 2 minutes of UV exposure (P = 0.0006). Thus, the relative decrease in progeny virus formation of the p28$^-$ mutant EV virus, increases with the duration of UV exposure. This observed difference in UV sensitivity between EV-WT and the p28$^-$ mutant EV infected cells is not due to incomplete or delayed virus infection with the p28$^-$ mutant EV virus since in the absence of UV treatment, no difference in the replication of EV and the p28$^-$ mutant EV virus was found (P = 0.43).

This agrees with previous studies that found no difference in replication between EV-WT and the p28$^-$ mutant EV following infection of a variety of cell culture lines

(Senkevich *et al.*, 1994; Senkevich *et al.*, 1995) and our observation that overexpression of SFV N1R in VV-infected cells does not affect replication. Results presented here have shown that UV treatment of HeLa cells infected with the p28$^-$ mutant EV results in apoptosis, whereas cells infected by EV-WT are protected. This data suggests that the reduced virus yield of the p28$^-$ mutant EV following exposure to UV may be a manifestation of inefficient apoptosis suppression by the p28$^-$ mutant EV rather than a direct requirement for EVp28 in promoting virus replication.

DISCUSSION

Self-destruction of virus infected cells by induction of apoptosis is an important host defense process, so it should not be surprising that many viruses have evolved mechanisms to avoid or delay its onset (Teodoro and Branton, 1997). Poxviruses block apoptosis at several distinct steps in the induction pathway. Well characterized examples include soluble TNF binding proteins that modulate activation of the TNF receptor 1 apoptotic signaling pathway (Sedger and McFadden, 1996) and the serpin encoded by *crmA* that inhibits cytotoxic-T-lymphocyte mediated apoptosis (Fas- and TNF induced) by blocking caspase activity (Zhou *et al.*, 1997). MCV possesses two genes (MC159L and MC160L) that contain duplicated death effector domain (DED) motifs that are found in proteins in the FAS and TNF signal transmission pathways (Senkevich *et al.*, 1997). Expression of MC159L has been shown to protect cells from apoptosis induced by these ligands (Bertin *et al.*, 1997).

The results presented here show that overexpression of SFV N1R gene in a recombinant VV significantly reduced apoptosis of cells infected with this virus compared to cells infected by wild type VV. This suggests that this protein may be involved in yet another poxviral anti-apoptotic process. At early times, induction of apoptosis is expected to inhibit the accumulation of infective virus progeny, at late times it may serve to interfere with the release and spread of the new virus particles. Our results indicate that the SFV N1R protein reduces apoptosis at late times (after 24 hr), thus it may serve to increase the spread of the virus infection in an infected animal. Additionally, both SFV-N1R and EVp28 proteins protected virus infected HeLa cells from UV induced apoptosis, but not from apoptosis induced through TNF ligand or anti-Fas antibody. This result not only indicates a specific role for this poxviral protein in modulation of UV induced apoptosis pathways, but further supports the idea that the molecular pathways involved in UV induced apoptosis are at least distinct from those involved in Fas or TNF induced apoptosis (Green, 1998).

Interestingly, a number of other proteins that possess RING motifs have been shown to be involved in the regulation of apoptosis. Baculoviruses encode an inhibitor of apoptosis (IAP) (Crook *et al.*, 1993) and several eukaryotic homologs have been cloned and sequenced (Uren *et al.*, 1996). The mammalian IAP homolog (MIH) B has been shown to bind TNF receptor-associated factor TRAF2 (Uren *et al.*, 1996) which also contains a RING motif (Rothe *et al.*, 1994) suggesting that the IAPs may interfere with signaling pathways required for apoptosis. In this respect, a C-terminal RING motif truncation mutant of MIH-B inhibits NF-κB induction by TNF and enhances TNF killing (Chu *et al.*, 1997).

In addition, immunoblot analysis indicates EVp28 acts upstream of caspase-3 since it suppresses caspase-3 activation in response to UV treatment. Thus, EVp28 likely functions during the UV signaling phase of apoptosis at or before caspase activation. UV irradiation has been shown to promote cytochrome c release from mitochondria in coordination with caspase-3 activation and these events are prevented by Bcl-2 expression (Kluck *et al.*, 1997). It will therefore be interesting to determine if EVp28 infringes on mitochondrial/Bcl-2 apoptotic pathways. Additionally, it is also evident from our apoptotic assays that VV and EV infection protect the infected cell from apoptosis induction by TNF, anti-Fas and UV light. This is a testament to the combined efforts of several documented poxviral anti-apoptotic proteins to target various control molecules in apoptosis pathways (for an excellent review, see McFadden and Barry, 1998).

Whether p28 is directly anti-apoptotic in response to UV induced pathways is, however, currently unknown, since we have been unable to express this protein in tissue culture cells in the absence of viral infection. As such, it remains questionable whether the protection from UV induced apoptosis observed here plays a role in protecting the virus infected cell from environmental UV damage *in vivo*, or whether yet unidentified EVp28 functions transverse UV induced molecular apoptotic pathways. It is, however, interesting that the defect in virulence of the p28$^-$ mutant EV, resulted in a failure of the mutant virus to

spread from the skin, a major portal of poxvirus entry in natural infections (Buller and Palumbo, 1991), and notably the primary organ affected by UV light (Griffiths *et al.*, 1998).

It is possible that this poxviral RING finger protein may promote replication and spread of the virus in the skin, by protecting the infected cell and or viral DNA from the effects of UV light. EV infection and pathogenicity in mice has previously been shown to be affected by environmental conditions (Roberts, 1964a) and both the identification of a cyclobutane pyrimidine dimer photolyase encoded by Melanoplus sanguinipes entomopoxvirus (Afonso *et al.*, 1999) and a novel MCV anti-oxidant selenoprotein (Shisler *et al.*, 1998) which protects cells from UV or peroxide induced damage suggests that poxviruses have indeed evolved to cope with, and are subject to environmentally induced genotoxic stress.

The EV RING finger protein has been described as being critical for the replication of this virus in resident murine peritoneal macrophages (Senkevich *et al.*, 1995), cells that are prone to significant inherent apoptosis levels (Papadimitriou *et al.*, 1980; Papadimitriou and van Bruggen, 1993). However, several EV strains that are attenuated *in vivo*, also undergo restricted replication (Roberts, 1964b) in these cells, as does VV (Natuk and Holowczak, 1985). It is striking that both reports of abortive virus infection of these macrophages resulted in detection of some early viral protein expression but undetectable viral DNA replication (Natuk and Holowczak, 1985; Senkevich *et al.*, 1995). The reported defect in replication of the p28$^-$ mutant EV virus may be indicative of apoptosis of the infected macrophages, since the VV study reported that the VV infected macrophages underwent a marked generalized cytopathic effect, becoming highly vacuolized, granular, rounded and detached from the substratum, in effect, displaying symptoms of apoptosis (Natuk and Holowczak, 1985). Clearly, inefficient viral suppression of apoptosis of these infected cells would lead to the observed diminished viral replication of the p28$^-$ mutant EV reported in these macrophages.

Since EVp28 is not required for replication of EV in tissue culture and the gene is absent from VV-Copenhagen, it is difficult to conceive a direct role for this protein in poxviral replication. Although the DNA-binding activity of this group of poxviral proteins may be required as an accessory factor for DNA replication or transcription in certain cell types, it is also possible that the proposed requirement of these poxviral RING finger proteins for viral replication stems from a role in apoptosis suppression such as has been proposed for the baculovirus apoptosis inhibitor p35 (Lu and Miller, 1995). It is proposed that p28 facilitates replication of the virus by protecting the infected cell from undergoing apoptosis. This hypothesis is supported by findings reported here, which show reduced yields of the p28$^-$ mutant EV compared to EV-WT following UV treatment of virus infected HeLa cells. Since poxvirus infection probably suppresses the expression of cellular UV protective genes, the synthesis of a virally encoded UV protective molecule may provide a selective advantage in those infected cells that are subject to UV stress *in vivo*. In this respect, several documented correlations between poxviral encoded anti-apoptosis proteins and virulence *in vivo* have been documented (Barry *et al.*, 1997; Messud-Petit *et al.*, 1998; Mossman *et al.*, 1996; Sedger and McFadden, 1996).

There is a positive correlation between protection from UV induced apoptosis by EVp28 and localization of this viral protein to the viral DNA replication factories, since the p28$^-$ mutant EV synthesizes a non-factory associated RING truncated p28 protein (Senkevich *et al.*, 1995). It is noteworthy that the UV wavelengths (peak 302nm) used in our experiments lie within the UV-B region, which is generally believed to exert cytotoxic effects through direct DNA damage, primarily the formation of cyclobutane pyrimidine dimers, 6-4 photoproducts, and thymine glycols (Griffiths *et al.*, 1998) and that SFV-N1R binds to DNA cellulose. EVp28 might may play a direct role in DNA damage repair or alternatively, it is tempting to speculate that the poxviral RING finger proteins, through binding and sequestering viral DNA, may somehow prevent the activation of cellular DNA damage sensors such as p53. This may be particularly important because of the cytoplasmic

nature of poxviral replication intermediates. In such a scenario, EVp28 may function analogously to the VV E3L ds RNA binding protein which inhibits apoptosis through blocking activation of interferon-induced protein kinase by dsRNA (Kibler *et al.*, 1997; Rivas *et al.*, 1998).

No close homologs of the poxviral RING finger protein have been found in database searches. Therefore the progenitor gene for these proteins remain obscure, however, it is likely that it was acquired by an ancestral poxvirus from a host cell and is reminiscent of a cellular factor which may play a role in apoptosis signaling. Although p28 contains a carboxy terminal RING finger motif, it is not a homolog of the baculovirus inhibitor of apoptosis protein, IAP (Crook *et al.*, 1993), as p28 lacks the baculovirus IAP repeat sequences.

Although the molecular pathways involved in UV induced apoptosis have remained largely fragmented, they are of considerable interest as epidemiological evidence strongly suggests a link between UV irradiation from sunlight and epidermal neoplasms (Brash *et al.*, 1996; Elder, 1989; Rundel and Nachtwey, 1978). Future research on this poxviral RING finger virulence protein family may prove a useful tool to help elucidate the precise nature of these apoptotic processes.

CONCLUDING DISCUSSION

The SFV N1R RING finger protein and several other poxviral homologs are known to localize to the sites of viral DNA replication, known as virus factories or virosomes within the cytoplasm of infected cells. This body of work has identified a DNA binding activity for N1R, suggesting a mechanism for localization to the virus factories. Immunofluorescence analysis of deletion and site specific SFV N1R mutant proteins transiently expressed in VV infected BGMK cells have led to the identification of sequence requirements for both factory localization and DNA binding activity. Although deletion mutagenesis initially identified a small region of the RING finger of N1R that was required to permit normal factory localization, the RING finger is not likely to govern the specificity of localization since site specific mutagenesis studies have identified a critical role for a conserved N-terminal region of N1R that is also required for both DNA binding and factory localization. Structural prediction and homology analysis have further indicated that N1R is a potential member of the ribbon-helix-helix family of DNA binding proteins that utilize a short beta sheet for DNA recognition. In support of this, mutation of the central beta strand residue of N1R, an invariant Asn residue (Asn 26) was found to be important for both factory localization and interaction with DNA. Future studies should be directed towards an evaluation of sequence specific DNA binding activity by N1R. Additionally, as members of this ribbon-helix-helix DNA binding family generally function as dimers, studies to determine the true oligomeric nature of N1R should be instigated.

The most significant contribution of this research is the finding of a role for SFV N1R and the EV virulence factor, p28, in protection from apoptosis. Overexpression of SFV N1R in VV infected cells was found to delay host cell apoptosis following VV infection and studies using known apoptotic inducers have pointed to a role for the poxviral proteins in protection from UV induced apoptosis, signaling upstream of caspase-3 activation. The

precise mechanism of apoptosis inhibition, however, remains to be more clearly defined. Whether the poxviral RING fingers proteins act as environmental viral genotoxicity protectors or act along UV induced apoptosis signaling pathways common to other undefined cellular functions remains unknown. There is a positive correlation, albeit weak, between factory localization and DNA binding activities of these viral proteins and apoptosis suppression since the p28-mutant EV virus used in studies presented here, expresses a RING finger truncated protein that fails to localize to the viral factories (Senkevich *et al.*, 1995).

The relationship between the role of SFV N1R in factory localization and inhibition of apoptosis is presently unknown, but it is not uncommon for viral gene products to have more than one function. Interestingly, in this respect, the baculovirus p35 protein, a broad spectrum inhibitor of apoptosis, was also found to facilitate viral replication (Hershberger *et al.*, 1992; Hershberger *et al.*, 1994). Future research on the role of the constructed VV-N1RAsn26ΔAla recombinant virus in apoptosis protection should provide a clearer definition of the requirement of DNA binding and factory localization for apoptosis protection.

A large body of recent evidence points to a role for RING finger motif containing proteins in the modulation of ubiquitination reactions (Freemont, 2000; Joazeiro and Weissman, 2000), It currently appears that RING fingers may act as E3 ubiquitin ligases and target selective proteins for degradation by the ubiquitin machinery. It is interesting that the EVp28 RING finger motif is implicated in apoptosis suppression, as the p28 mutant EV used in this apoptosis study expresses a RING finger truncated protein. Whether N1R or EVp28 also act as ubiquitin ligases remains to be experimentally determined. However, it is tempting to speculate that the observed apoptosis protective ability of these poxviral proteins could result from targeted degradation of a critical cellular factor required for apoptosis signaling in response to DNA damage. Clearly, future research into this working hypothesis is warranted.

The finding of a role for the EV virulence factor, p28, in apoptosis protection and studies on the production of EV progeny virus following UV exposure of EV infected HeLa cells indicates the previously reported requirement for EVp28 in promoting replication of EV in mouse peritoneal macrophages cells (Senkevich *et al.*, 1995) may be reflective of inefficient viral suppression of host cell apoptosis by the p28-mutant EV following infection of these apoptosis prone cells. While the correlation between apoptosis suppression, viral replication and virulence is not new to the area of poxvirus research (McFadden, 1998; McFadden and Barry, 1998), the finding of a role for these poxviral RING finger proteins in protection from UV induced apoptosis is novel. Future research on these poxviral RING finger proteins may shed light on the control of cell survival following UV irradiation. Such information is essential for the development of strategies, either peptide or pharmacologically based, to increase the sensitivity of transformed cells to the effects of DNA damage induced therapy, currently a major obstacle to the effectiveness of current cancer therapies.

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