Characterization of Orthoreovirus Core Proteins By Sequencing and Proteomic Analysis of Avian Reovirus μ Proteins and Development of α-Mammalian Reovirus Core Protein Monoclonal Antibodies

By

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of

Master of Science

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THE UNIVERSITY OF MANITOBA

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

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List of Abbreviations

a.a.	amino acids
ACN	acetonitrile
ARV	avian reovirus
ATPase	adenosine triphosphatase
BCA	bicinchoninic acid
BRV	baboon reovirus
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CPE	cytopathic effect
dATP	deoxyadenosine triphosphate
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphatase
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
HO Buffer	homogenization buffer
Ig	immunoglobulin
IgG	immunoglobulin G
ISVP	intermediate/infectious subviral particle
L929	mouse fibroblast cell line
MALDI QqTOF	matrix-assisted laser desorption/ionization quadrupole time of flight
MEM	minimal essential medium
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MRV	mammalian reovirus
MS	mass spectrometry/spectrometer
MS/MS	tandem mass spectrometry
m/z	mass/charge ratio
NBV	Nelson Bay reovirus
NTPase	nucleotide triphosphatase
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PI	post infection
QM5	continuous quail cell line
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNase	ribonuclease
RRV	reptilian reovirus

RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ss	single-stranded
T1L	type 1 Lang
T2J	type 2 Jones
T3D	type 3 Dearing
TBST	Tris-Buffered Saline + Tween
TFA	trifluoroacetic acid
TGU Gel	Tris-Glycine-Urea Gel
TOF	time of flight
VSP	viable serum protein
v/v	volume per volume
w/v	weight per volume

Abstract

Reovirus is a double-stranded RNA virus with a segmented genome. The genome consists of 10 RNA segments which encode 8 structural and 3 non-structural proteins. The virus exists in three forms: virus, infectious/intermediate subviral particle and core. The prototype reovirus is the mammalian reovirus (MRV); however, other types of reoviruses exist including avian (ARV), reptilian and aquareoviruses. ARV and MRV were the two types of virus used in this study. The focal points of this investigation were the reovirus core proteins. The core consists of five proteins, named λ1, λ2, λ3, μ2 and σ2 in MRV. The homologous core proteins in ARV are λA, λB, λC, μA and σA. In order to learn more about these proteins, sequencing of the ARV M1 gene and analysis of its predicted μA protein was undertaken, as well as the other ARV M genes and μ proteins in an attempt to advance the sequencing of the ARV genome. A different aspect of this investigation involved the production and characterization of α- MRV core protein antibodies which can be used as molecular tools to enable future studies of the structure and function of the core proteins.

In the first portion of this study, the M1, M2 and M3 genes and the predicted μA, μB and μNS proteins of ARV 138 and 176 were sequenced and analyzed. Traditional dideoxynucleotide sequencing was used to obtain the initial sequence data. Further proteomic analysis was then performed on the μA and μB proteins to ensure that the sequence lacked insertions and deletions. Computer analysis was performed to predict and characterize the secondary structure, hydropathy, buried residues and isolectric points of each ARV μ protein. The ARV μ proteins were compared with the homologous MRV proteins, as well as aquareovirus proteins identified by BLAST searches in the cases of

μA and μB, to identify highly conserved regions and amino acids which may play important structural and functional roles of the proteins.

The other half of this study involved the characterization of MRV core specific monoclonal antibodies. Monoclonal antibodies were produced by the injection of dissociated cores and the specificity of the antibody was determined afterwards. Eleven monoclonal antibodies were initially identified as ELISA positive; of these, only two (#2-5C2 and #2-3C11) were Western Blot Positive. The specificities of #2-5C2 and #2-3C11 were identified by the elution of the MRV λ1 and λ2 proteins from a Tris-Glycine-Urea Gel and analysis by Western Blot. Both antibodies were specific for the λ2 protein. An immunoprecipitation experiment linked with mass spectrometry was used in the initial characterization of Western Blot negative antibody, #1-3C11. The experimental conditions were optimized and the antibody pulled down λ1, λ2, and σ2. Immunoprecipitation linked with in-gel digestion also identified an association between #1-3C11 and λ1 and σ2. It remains unclear whether a complex between these proteins is formed or whether the protocol needs to be optimized and further work is required to complete the characterization of this antibody. This study has characterized two α-reovirus λ2 monoclonal antibodies that may now be used as reagents in a wide variety of assays.

1. Introduction

1.1 Reovirus

Infectious diseases caused by RNA viruses represent approximately 90% of all cases of infectious disease throughout the world (WHO, 1996). Poliovirus, Measles virus and Influenza viruses are all examples of RNA viruses which have caused significant human disease. The economic burden of viral infections is huge when considering workdays lost, medical treatment and family stresses (Murray and Lopez, 1996). Children are particularly susceptible to viral infections. The mechanisms of viral pathogenesis, virus-host cell interactions and viral functioning must be elucidated before effective anti-viral treatments can be developed and virus transmission can be limited.

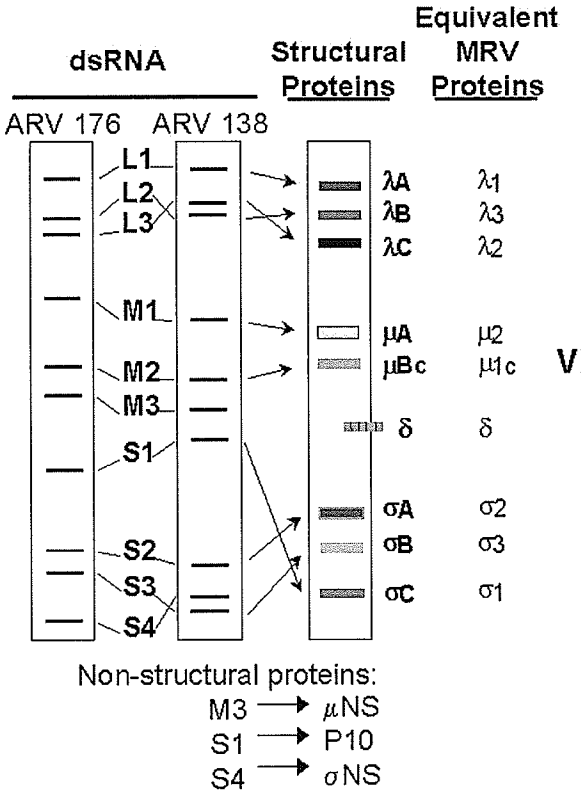
Mammalian reoviruses (MRV), as well as avian reoviruses (ARV), reptilian reoviruses (RRV), Nelson Bay reoviruses (NBRV) and Baboon Reoviruses (BRV), comprise the genus *Orthoreovirus* in the family *Reoviridae*, with MRV as the prototype members. The acronym *reo* within the name reovirus stands for respiratory and enteric orphan viruses, indicating the viruses are capable of infecting both respiratory and enteric systems without serious pathogenesis in human hosts (Sabin, 1959). The majority of identified reoviruses are not pathogenic in humans; however, Rotaviruses are responsible for a large number of viral-induced deaths, primarily in children, and are a large health concern, especially in underdeveloped countries. Reoviruses are predominantly found in a wide variety of water sources, which is a likely route for viral dissemination.

Because the majority of reoviruses are not pathogenic in humans, with the exception of immunocompromised patients, they represent a useful tool for the study of viral pathogenesis and molecular function. MRV are extremely well characterized, safe, easy

to culture and grow relatively quickly. MRV are a useful tool for the study of non-enveloped viral entry and pathogenesis as all non-enveloped viruses likely use similar mechanisms. Several important discoveries that have been made using MRV include receptor mediated endocytosis (Silverstein and Dales, 1968), 5' mRNA capping (Furuichi *et al*, 1975) and Kozak's sequence (Kozak, 1982a, Kozak, 1982b).

1.2 Reovirus Structure

The majority of identified reoviruses, including the prototype MRV and ARV, contain 10 double stranded RNA (dsRNA) genome segments surrounded by a non-enveloped, icosahedral double capsid of 70-80 nm in diameter (Figure 1). The mammalian reovirus genome segments are classified into three groups based on their electrophoretic mobilities: 3 large segments (L1, L2, L3), 3 medium segments (M1, M2, M3) and 4 small segments (S1, S2, S4, S4) which code for the proteins λ3, λ2, λ1, μ2, μ1, μNS and μNSC, σ1 and σ1s, σ2, σNS and σ3, respectively (Shatkin *et al,* 1968; McCrae and Joklik, 1978; Mustoe *et al.*, 1978b). Like the prototype mammalian reoviruses, avian reovirus genome segments are also classified by their electrophoretic mobilities: 3 large segments (L1, L2, L3), 3 medium segments (M1, M2, M3) and 4 small segments (S1, S2, S4, S4) which code for the proteins λA, λB, λC, μA, μB, μNS, σ3, σ1, σ2 and σNS, respectively in ARV (Varela *et al.*, 1994). The mammalian reovirus particle is composed of an inner and outer capsid (Figure 1). The outer capsid consists of 600 copies each of the μ1 and σ3 proteins and 36 copies of the σ1 protein. The inner capsid, termed the core, consists of 120 copies of the λ1 and 150 copies of the σ2 protein composing the inner shell and 12 λ2 pentamers that form spikes at the icosahedral vertices of the particle. The minor copy proteins, λ3 (present in 12 copies), the


dsRNA
Structural Proteins
Equivalent MRV Proteins
ARV 176
ARV 138
L1
L2
L3
M1
M2
M3
S1
S2
S3
S4
λA
λB
λC
μA
μBc
δ
σA
σB
σC
λ1
λ3
λ2
μ2
μ1c
δ
σ2
σ3
σ1
Non-structural proteins:
M3 → μNS
S1 → P10
S4 → σNS

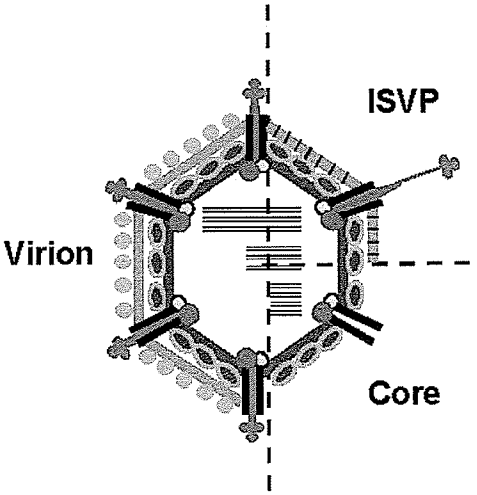

ISVP
Virion
Core

Figure 1. Coding assignments and locations of structural proteins within reovirus particles. The reovirus genome consists of ten dsRNA segments, classified by size as the L, M and S segments which encode the λ, μ, and σ proteins, respectively. Diagrammatic cartoons of a dsRNA gel, a protein gel and a composite particle. Proteins in the protein gel and composite particle are colour coded with gene ⟶ protein assignments are indicated by arrows. Only the structural proteins are represented here.

RNA-dependent RNA polymerase (RdRp) and μ2, the hypothesized RdRp cofactor (present in 20 copies), are found on the interior of the core particle.

In nature, reoviruses exist in three forms: the complete virion, the ISVP (infectious or intermediate subviral particle) and the core particle (Figure 1). Complete virions are proteolytically processed to generate the other two forms of the virus. The virus may be degraded extracellularly by proteases, i.e. trypsin, pepsin, chymotrypsin (Joklik, 1972; Borsa *et al.*, 1973) in the intestinal tract into ISVPs. This conversion results in the cleavage of μ1 into δ and φ, loss of σ3 and the extension outwards of the σ1 proteins. ISVPs can be further proteolytically digested, with the degradation of φ, δ, and σ1 proteins. This further proteolysis results in a conformational change in the λ2 pentamers found at the vertices of the particle (Dryden *et al.*, 1993) and results in the core form of reovirus. ISVPs and cores can be generated *in vitro* (Mayor and Jordan, 1968); ISVPs are infectious, yet not transcriptionally active while core particles are not infectious but transcriptionally active.

1.3 Reovirus Life Cycle

The reovirus life cycle can be divided into three stages: 1- infection and viral uncoating 2- transcription, translation and replication, 3- viral assembly. A general overview of the reovirus life cycle can be found in Figure 2. Reovirus infection is primarily enteric; the virus usually enters a new host by the fecal-oral route. Upon entering the host, the virus can infect cells in either the respiratory or the enteric system. A virus or ISVP can enter a host cell by two distinct routes. In either case, the virus must first bind to a cellular receptor before internalization. The predominant cellular receptor which reovirus binds to is the junction adhesion molecule (JAM-1) (Barton *et al.*, 2001),

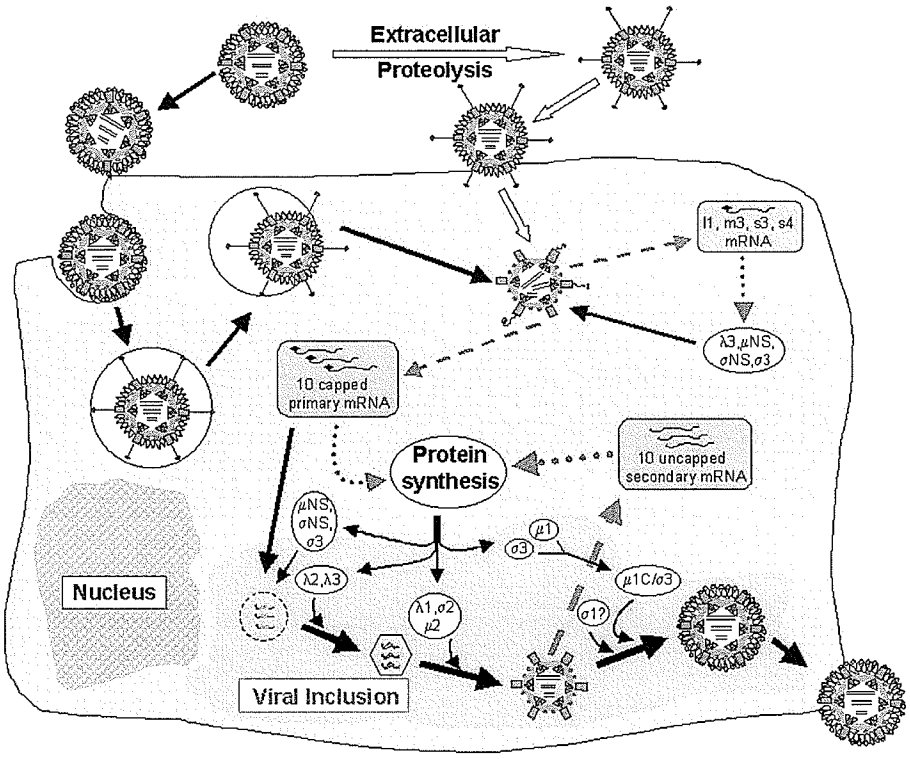

Extracellular
Proteolysis
l1, m3, s3, s4
mRNA
λ3,μNS,
σNS,σ3
10 capped
primary mRNA
Protein
synthesis
10 uncapped
secondary mRNA
μNS,
σNS,
σ3
μ1
σ3
λ2,λ3
μ1C/σ3
λ1,σ2
μ2
σ1?
Nucleus
Viral Inclusion

Figure 2. Mammalian Reovirus Life Cycle. This cartoon represents the replication cycle of a reovirus particle within a host cell. The virus binds to the cell surface via a receptor and is internalized by receptor-mediated endocytosis (upper left). Inside the endocytic vesicle the virus is exposed to proteolytic enzymes which cleave and unfold the outer capsid proteins; this results in the formation of the intermediate/infectious subviral particle (ISVP). The ISVP uncoats further as it leaves the endocytic vesicle, forming the core particle which is responsible for viral transcription and translation. Alternatively, the virus may be exposed to extracellular proteases, forming an ISVP and the ISVP can directly infect the cell, bypassing the endocytic pathway (top centre). The ISVP uncoats inside the cell, revealing the core particle. The core particle transcribes four primary viral RNA segments which are then translated to produce proteins to further activate the core. The core then produces a full set of primary mRNA which is translated to produce the primary proteins. Viral inclusion bodies are formed and it is within these bodies that viral proteins and mRNA segments come together to form new core particles (bottom centre). These newly formed cores produce secondary mRNA. The process of translation and assortment continues and when the host cell is no longer able to support virus production the cell is lysed and the new virus particles are released from the cell (bottom right). This figure was adapted from Reoviruses (Coombs, 2002).

although studies have shown the virus is also able to bind to sialic acid, sialoglycoproteins and the EGF receptor (Gentsch and Hatfield, 1984; Paul *et al*, 1989; Choi *et al*, 1990; Tang *et al.*, 1993). The virus protein responsible for cellular attachment is the σ1 protein, and the interaction of the virus and host proteins induces a conformational change in the viral capsid which is required for viral entry. This conformational change increases the particle's sensitivity to proteases. Once the virus is attached to the host cell, the virion or the ISVP may undergo receptor-mediated endocytosis into clathrin coated pits (Borsa *et al.*, 1979; Rubin *et al.*, 1992). ISVPs, which can be produced extracellularly by proteases, may bypass the receptor mediated endocytosis route of entry and enter the cell by direct passage across the membrane (Borsa *et al.*, 1979). The entry of ISVP particles into the host cell is acid independent. Once the virus or ISVP has entered the cell it is proteolytically cleaved until core particles are produced.

Once a core particle has made its way to the cytoplasm, transcription can begin. Cores are transcriptionally active, containing the RdRp, as well as a hypothesized cofactor. The reovirus RdRp is the λ3 protein (Drayna and Fields, 1982; Starnes and Joklik, 1993) and the putative cofactor is the μ2 protein (Wiener *et al.*, 1989; Yin *et al.*, 1996). Functional copies of both proteins are required for transcription to occur. The RdRp produces 5' capped, non-polyadenylated messenger RNA (mRNA). The λ3 protein synthesizes mRNA from each of the ten genomic strands during primary transcription. The newly formed viral mRNA molecules are then translated by the host ribosomes into protein. These viral proteins associate with primary mRNA transcripts into full

complements of genes and structural proteins without the outer capsid and the λ2 core protein. The mechanism by which this packaging occurs remains unclear.

The packaged mRNA transcripts serve as templates for the minus-strand synthesis by the RdRp to complete the dsRNA genome. The packaged particles now contain dsRNA genomes and are able to mediate the secondary transcription process, by which the majority of the mRNA transcripts of the infection are produced. The secondary transcripts can easily be distinguished from the primary transcripts, as they remain uncapped (Zweerink *et al.*, 1972). It is not known whether secondary transcripts are packaged into progeny virions and used for minus-strand synthesis or whether they are only used for translation into proteins.

The process of completing viral assembly by the addition of the outer capsid remains poorly understood. It is thought that the first step in the assembly pathway involves mRNA in complex with the non-structural proteins, μNS, σNS, as well as the structural protein σ3 (Antczak and Joklik, 1992). The next form of the particle, "replicase particles", appears to have lost the non-structural proteins and include both inner and outer capsid proteins (Morgan and Zweerink, 1974). This particle is involved in minus-strand synthesis. More copies of the outer capsid proteins are added to the replicase particle to form complete viral particles (Morgan and Zweerink, 1974; Zweerink *et al.*, 1976). Group B and Group G temperature sensitive (ts) mutants produce core-like particles at the non-permissive temperature. Group B ts mutants are mutated in the L2 gene which encodes the λ2 protein (Mustoe *et al.*, 1978a) while the mutation in Group G ts mutants is located in the S4 gene (Cross and Fields, 1972) which encodes the σ3 protein. These particles resemble cores in that they contain 10 dsRNA genome segments,

contain the four structural core proteins, have the same buoyant density of cores and have the same diameter. Group B ts mutants synthesize a normal amount of the λ2 protein; however, they are unable to condense the core particles with the outer capsid proteins to form complete particles (Morgan and Zweerink, 1974). Group G ts mutants also form core-like particles as interaction between σ3 and μ1 is required for condensation of the outer capsid proteins with nascent cores (Shing and Coombs, 1996). This suggests that particle assembly may be a two step process with a minor amount of λ2 required to commence assembly, with the majority of the protein added later in the process (Morgan and Zweerink, 1974). Viral egress is mediated by the death of the host cell and breakdown of its cellular membrane, resulting in cell lysis and virion release. Reovirus-induced apoptosis appears to be the primary mechanism by which host cell death occurs (Tyler *et al.*, 1995; Tyler *et al.*, 1996, DeBiasi *et al.*, 2003).

1.4 MRV vs. ARV: Similarities and Differences

There are many similarities and differences between the two species, ARV and MRV. Like the better-studied MRV, ARV is a non-enveloped icosahedral particle with a 70-80 nm diameter and carries 10 dsRNA linear gene segments. Although the crystal structure of ARV has not yet been determined, morphologically ARV and MRV appear similar under the electron microscope. ARV have a number of unique features associated with them that differentiate them from MRV. These include a different host range than MRV, lack of hemagglutination, and ability to induce syncytia in cell culture (Martinez-Costas, 1997; Sheng Yin, 2000). ARV serve as the prototype of all syncytia-inducing non-enveloped viruses (Duncan, 1996) and a novel fusogenic protein (p10) associated with this behaviour has been identified (Shmulevitz and Duncan, 2000). Proteins with similar

conserved regions and motifs have been identified in NBV and BRV (Shmulevitz and Duncan, 2000) and all non-enveloped viruses capable of forming syncytia belong to the *Reoviridae* family. Genomic coding similarities exist between the MRV and ARV although there are exceptions. For example, the ARV S1 genome segment encodes the ARV σ3 protein, while in the MRV system the structurally analogous protein is the σ1 protein.

While the MRV replication cycle has been well characterized, the ARV replication cycle has yet to be fully clarified. The similarities in protein structure, function and distribution suggest that the replication cycles are also similar. Many of the ARV proteins involved in replication have not been fully characterized, leaving gaps in the understanding of its replication cycle. For example, the MRV σNS protein binds single stranded RNA (ssRNA), is involved in forming viral inclusion factories and may be involved in secondary transcription and assortment (Miller *et al,* 2003). The ARV σNS protein binds ssRNA (Yin and Lee, 1998; Sheng Yin, 2000) however; its role in assortment and transcription remains unclear.

1.5 The MRV μ proteins

In MRV, the M1 gene encodes the μ2 protein which is hypothesized to be an RdRp cofactor (Table 1). The M1 gene has been shown to be associated with the development of murine myocarditis following reovirus infection (Sherry *et al.*, 1998), as well as with strain specific growth differences in cultured mouse heart cells (Matoba *et al.*, 1991), bovine aortic endothelial cells (Matoba *et al.*, 1993) and MDCK cells (Madin-Darby canine kidney epithelial) (Rodgers *et al.* 1997). The biochemical properties of the μ2 protein have been well characterized and the complete protein sequence has been

Table 1- Properties of Mammalian Reovirus Proteins

Gene Segment	Protein	Mass (kDa)	Copy No.	Location in Virions	Function or Property
L1	λ3	142	12	inner capsid	RNA-dependent RNA polymerase
L2	λ2	145	60	outer capsid	guanylyltransaferase methyltransferase
L3	λ1	143	120	inner capsid	binds RNA, Zn metalloprotein, NTPase? RNA helicase?
M1	μ2	83	20	inner capsid	binds RNA, NTPase
M2	μ1	76	600	outer capsid	role in penetration/transcription activation
M3	μNS	80	0	nonstructural	binds core, role in secondary transcription/RNA assortment or replication
	μNSC	75	0	nonstructural	unknown
S1	σ1	49	36	outer capsid	cell attachment protein, hemagglutinin
	σ1s	14	0	nonstructural	dispensable in cell culture
S2	σ2	47	150	inner capsid	binds dsRNA
S3	σNS	41	0	nonstructural	binds ssRNA, role in RNA assortment or replication
S4	σ3	41	600	outer capsid	binds dsRNA, Zn metalloprotein, effects on translation

determined from a wide variety of viral isolates (Wiener *et al.*, 1989, Zou and Brown, 1992, Parker *et al.*, 2002, Yin *et al.*, 2004). The μ2 protein is a minor component of the inner capsid, present in 20-24 copies per virus particle (Coombs, 1998). It is believed that μ2 is located inside the core particle, at the vertices of the icosahedron, however, this has not yet been clearly demonstrated. *In vitro* studies have shown that the protein interacts with the λ3 protein, the RdRp, as well as functioning as an NTPase, RNA 5' triphosphatase and RNA-binding protein (Brentano *et al.*, 1998, Kim *et al.*, 2004,). The μ2 protein has also been shown to induce interferon production (Sherry and Blum, 1994; Sherry *et al.* 1998). It is found in viral inclusion bodies during replication, as well as in the cytoplasm and nucleus of the host cell in small amounts. Lesions in the M1 gene prevent the formation of viral inclusions (Coombs, 1996), suggesting that the μ2 protein may be involved in an intermediate step of virion assembly. Studies have also indicated that μ2 may be associated with viral plaque size (Moody and Joklik, 1989).

The M2 genome segment in MRV encodes the μ1 protein (Table 1). The M2 genome segment from a number of different isolates has been sequenced and the corresponding protein has been analyzed (Tarlow *et al.*, 1988, Jayasuriya *et al.*, 1988). The μ1 protein is a major outer capsid protein and present in high copy number (600 per virion). During assembly, μ1 undergoes assembly-mediated proteolysis, resulting in the μ1C fragment, which is the predominant form of the protein, found in the mature virion (Zweerink and Joklik, 1970). μ1 is involved in virus penetration of the host cell, as well as transcription activation. As described above, following cellular penetration, μ1 is proteolytically cleaved during viral entry into φ and δ. The cleavage junction is surrounded by α-helices, which may interact with the cellular membrane, promoting viral entry. The μ1 protein

also appears to play a role in the regulation of the RNA transcriptase (Drayna and Fields, 1982); however, the mechanism by which μ1 performs this regulation remains unclear.

The μNS protein is encoded by the M3 genome segment in MRV. The role of μNS in reovirus infection has not yet been clarified (Table 1). The μNS protein is produced in relative abundance compared with other virally produced proteins (Joklik, 1983). μNS has been shown to interact with viral plus-strand RNA transcripts, suggesting that μNS may be involved in translation of the viral transcripts, synthesis of the minus-strand or the packaging and sorting of the RNA segments into new particles. μNS has been shown to associate with core particles (Broering, 2000) and virus assembly intermediates (Morgan, 1975), suggesting μNS may play a role in the regulation of virus transcription or assembly. While the function of μNS remains poorly understood, the sequence of the M3 genome and the μNS protein from several virus isolates (McCutcheon *et al.,* 1999; Wiener *et al.,* 1989) has been identified. The μNS protein contains a large number of α-helices, predominantly at the carboxyl-terminus, and has a tendency to form coiled coils (McCutcheon *et al.*, 1999).

1.6 Reovirus Core Proteins

The reovirus core particle is made up of five proteins: λ1, λ2, λ3, μ2 and σ2. Each protein is present in variable copy number and plays a unique role in the function of the core. As mentioned above, the core particle is unable to infect cells but has transcriptional activity. The virion and ISVP are proteolytically degraded into the core structure to expose and functionally activate the core proteins.

The λ1 protein, the major core capsid component, is encoded by the L3 gene segment in MRV and present in 120 copies per core particle, making it a relatively

abundant protein within the structure (Table 1). In the core particle, the λ1 protein forms dimers which assemble into the basic core structure. The remaining four core proteins are built on top and around these λ1 dimers (Kim *et al.*, 2002). However, *in vitro* studies have shown that the λ1 protein must be coexpressed with the σ2 protein into the icosahedral core particle (Xu *et al.*, 1993). As the major core capsid protein, the λ1 protein interacts with λ2 and σ2, as well as the other minor core proteins, to a lesser extent. The λ1 protein has a number of activities which play an important role in aiding the transcriptional activity of the core particle. Several studies have demonstrated that the λ1 protein has ATPase activity, is a zinc metalloprotein and is able to bind dsRNA (Lemay and Danis, 1994; Bisaillon *et al.*, 1997). The function of the λ1 protein has not well been characterized. Studies have shown that the protein may play a role in the capping of primary mRNA and unwinding the dsRNA during the early stages of infection (Bisaillon *et al.*, 1997). Interestingly, the ability of the λ1 protein to bind dsRNA is not limited to reovirus RNA and the zinc finger motif in the N-terminus of the protein plays no functional role in the binding of RNA (Lemay and Danis, 1994).

The λ2 protein is encoded by the L2 gene segment in MRV (Table 1). This protein is a structural protein whose pentamers form turret-like projections at each of the fivefold axes of the core particle. The λ2 protein interacts with both the inner and outer capsid structural proteins, but there is little evidence demonstrating whether it interacts with the minor, low copy number core proteins, λ3 and μ2. The λ2 protein is present in a moderate copy number, 60 copies of the protein per virion. Temperature sensitive mutants with lesions in the L2 gene which encodes the λ2 proteins are unable to form complete virions at the non-permissive temperature or may form spike-less core particles,

however, they are able to synthesize dsRNA and form core particles (Morgan and Zweerink, 1974; Mustoe *et al.*, 1978b). These results indicate that λ2 plays a role in outer capsid assembly. Studies have demonstrated that the λ2 protein has both guanylyltransferase and methyltransferase activity (Shatkin, 1974; Cleveland *et al.*, 1986). The guanylyltransferase activity of the λ2 protein is involved in mRNA capping of the primary viral transcripts (Cleveland *et al.*, 1986, Fausnaugh and Shatkin, 1990, Luongo *et al.*, 2000) and the methyltransferase activity may play a role in mRNA capping as well, although this process is not as well characterized (Shatkin, 1974; Luongo *et al.*, 1998).

The λ3 protein is encoded by the L1 gene in MRV and, as mentioned above, is the RdRp (Table 1). The λ3 protein sequence has homology to other RdRp sequences (Bruenn, 1991). When the λ3 protein is expressed *in vitro*, the protein demonstrates limited polymerase activity (Starnes and Joklik, 1993). Expression of solely the λ3 protein does not result in transcription however, suggesting that a cofactor must be required. Genetic reassortment studies have mapped transcriptase activity to the L1 gene which encodes the λ3 protein (Drayna and Fields, 1982). Group D ts mutants with mutations in the L1 gene have a dramatic reduction in dsRNA synthesis, and form empty particles at non-permissive temperatures. The λ3 protein is a low copy number protein; it is only found in 12 copies per virus particle and is thought to be located at the vertices of the icosahedron. Studies have shown that the λ3 protein interacts with λ1 proteins, λ2 proteins and μ2 proteins within the core particle (Nibert *et al.*, 1991; Kim *et al.*, 2004). Further reassortment studies of the L1 gene have also suggested that the λ3 protein plays a role in isolate-dependent differences in growth amongst a variety of different cell lines

(Matoba *et al.*, 1991; Rodgers *et al.*, 1997), as well as the induction of myocarditis in newborn mice (Sherry and Blum, 1994).

The M1 gene and its translation product, μ2, was discussed in section 1.5. Briefly, in MRV, the M1 gene encodes the μ2 protein which is hypothesized to be an RdRp cofactor. The μ2 protein is a minor component of the inner capsid, present in 20-24 copies per virus particle (Coombs, 1998). *In vitro* studies have shown that the protein interacts with the λ3 protein, the RdRp, as well as functioning as an NTPase and an RNA 5' triphosphatase (Kim *et al.*, 2004). Homology searches against protein sequence databanks show little to no homology between the MRV μ2 protein and other known protein sequences.

In MRV, the σ2 protein is encoded by the S2 gene segment (Table 1). The protein is a major component of the core capsid and is present in 150 copies per core particle. The σ2 protein interacts with the other major core capsid component, λ1, to form the core shell. Studies also suggest that σ2 binds to the μ1 major outer capsid protein, anchoring the core particle to the outer shell (Reinisch *at al.*, 2000). A temperature sensitive mutant with a lesion in the S2 gene segment fails to synthesize viral dsRNA or assemble replicase particles at the non-permissive temperature (Ramig *et al.*, 1978). Other studies have demonstrated that the σ2 protein has dsRNA binding activity (Schiff *et al.*, 1988). The information from these studies implies that the σ2 protein plays a role in either virion assembly or viral RNA synthesis. Further study into the structure and function of the σ2 protein is required to better understand its role within the core particle, as well as function during the viral life cycle.

1.7 Antibody General Overview

Antibodies are an important molecular tool, which are used in both the laboratory and clinical environment in a number of ways. Antibodies have unique binding specificity and are predominantly able to bind antigen towards which they have been specifically raised, either synthetically or within a host. However, many antibodies do demonstrate cross-reactivity with antigens of similar sequence or structure. Some of the tests that antibodies can be used in include Western Blots, immunoprecipitations, enzyme-linked immunosorbent assays (ELISAs), protein purification and radioimmunoassays. The wide variety and large number of uses for specific antibodies demonstrate the need for large-scale antibody production and characterization. The generation of hybridomas is a good way to generate a large quantity of specific antibody. Hybridomas can be generated by the injection of antigen into an animal, harvesting the spleen cells and fusion of the spleen cells with myeloma cells. Monoclonal antibodies can be produced by cloning of the hybridoma cells and ELISA can be used to identify the specific antibody produced. The antibody can then be used in a wide variety of assays in the laboratory. Besides the traditional usage in ELISA, Western Blots and immunoprecipitation, antibodies are now being used in a wide variety of other ways, including as probes in antibody-based microarrays (Huang, 2001) and in the identification of or use as biomarkers (Pitarch *et al.*, 2004; Sinclair *et al.*, 2004).

Antibodies, also known as immunoglobulins (Ig), are produced by B-lymphocytes in response to infection or challenge with an antigen. Generally, following infection or challenge, an antigen presenting cell engulfs the antigen, digests it and displays short peptides on its surface by way of major histocompatibility complex II.

Helper T lymphocytes which recognize the antigen then stimulate B lymphocytes to begin specific antibody production. The stimulated B lymphocytes undergo a series of repeated cell divisions, cell enlargement and differentiation to produce a set of B lymphocyte clones producing specific antibody which is used by the body to combat the infection or challenge and provide protein should the antigen be encountered again (Janeway, 2001). The primary function of antibodies is antigen binding; however, antibodies have a number of effector roles within the immune response. These include the fixation of complement to promote the lysis of cells and release of immune relevant molecules; the activation of other phagocytes, lymphocytes, platelets, basophils and mast cells as part of the immune response. As well, a class of antibodies function as neutralizing antibodies, in that they bind and inhibit the function of the antigen or protein (Janeway, 2001). There are five molecular classes of immunoglobulins; IgG, IgA, IgM, IgD and IgE (Barrett, 1988). As IgG is the class of antibody discussed in this work, it will be discussed further. IgG represents approximately 80% of the total antibody in human antiserum (Janeway, 2001). Each IgG molecule consists of two heavy and two light chains. The heavy chains are classified as γ and the light chain can be classified as either κ or λ. The two heavy chains and two light chains of IgG are linked by disulfide bonds that maintains the stability of the molecule. Papain cleavage of the IgG molecule results in three fragments; Fc and two identical Fab (Porter, 1973). The Fc fragment is unable to bind antigen and represents the carboxyl half of the two heavy chains bound together by at least one disulfide bond. Each Fab fragment consist of the amino terminal half of the heavy chain plus one light chain. It is the Fab fragments that are responsible for antigen binding and as such, each IgG molecule is able to bind two antigen molecules. Evidence

provided by papain cleavage of IgG suggests that the heavy chain is bent at the position where the antibody combines with the antigen (Porter, 1973). This suggests that the conformation of the antigen plays a vital role in antibody-antigen recognition. As mentioned above, each IgG is produced to target a specific antigen; however, the molecule may demonstrate cross-reactivity with antigens of similar shape and size.

1.8 Antibody Specificity Identification

Novel and efficient approaches are required for antibody specificity characterization if mixtures are used to induce antibody production within the original host mouse. Mass spectrometric-based approaches are quite useful in determining protein identification and can be used to determine the antigen binding to a specific antibody (Nelson *et al.*, 1995). In this approach, antibody with known specificity was used to capture antigen from human blood samples and mass spectrometry was then used to identify the antigen. Epitope mapping can also be performed with mass spectrometric-based approaches by the capture of an antigen with an antibody and digestion of the unbound antigen (Suckau *et al.*, 1990; Arockiasamy *et al.*, 2004) This allows for the identification of the portion of the antigen which is specifically bound to the antibody. Matrix-assisted laser desorption/ionization quadrupole time of flight (MALDI QqTOF) provides accurate analysis of a protein that has been fragmented into its peptide subunits (Figure 3). Protein samples can be prepared by a number of different methods, including antigen capture by an antibody, which all conclude with the proteolytic digestion of the protein; the enzymes primarily used are trypsin and the endoproteinase Glu-C. The peptide fragments are spotted onto a specially designed target and pulsed with a laser beam, which aerosolizes each fragment and gives them an ionic charge. Inside the

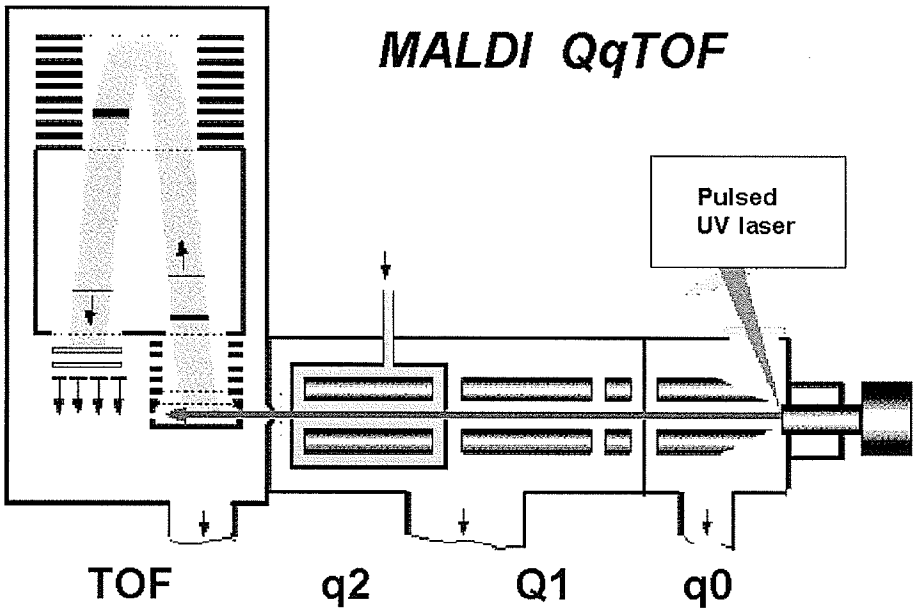

MALDI QqTOF
Pulsed
UV laser
TOF
q2
Q1
q0

Figure 3. The MALDI QqTOF mass spectrometer. The protein of interest is proteolytically cleaved and spotted onto a target which is then loaded into the machine. The sample is exposed to a laser beam and the individual peptides of the protein are carried through an electromagnetic field (q0, Q1 and q2) to the time-of-flight (TOF) analyzer. The amount of time required for a peptide to reach the TOF analyzer is proportional to its mass and charge. During tandem mass spectrometry, (MS/MS), a peptide of a specified mass and charge can be isolated and fragmented further in the q2 quadrupole. This allows for the analysis of the peptide amino acid sequence. The computer programs predominantly used to analyze the spectra produced by the MALDI QqTOF in this experiment were M/Z, Knexus and Sonar MS/MS (Proteometrics, New York, USA). This figure was provided by Dr. Werner Ens and Ken Standing (University of Manitoba, Department of Physics and Astronomy).

MALDI QqTOF instrument, the fragments are carried through an electromagnetic field towards the time of flight (TOF) analyzer. The analyzer is calibrated to determine the length of time that it takes each peptide fragment to travel a designated distance. The computer is then able to calculate the fragment's mass to charge ratio (m/z) based on the fragment's travel time. The m/z values of each digested protein can be compared with a database to determine the identity of the protein or determine whether the protein of interest has homology to other known proteins. The accuracy of the MALDI QqTOF has been determined to be <0.1 Da with respect to proteins which have molecular weights of between 500 and 4500 Da (Loboda *et al.*, 2000). When further analysis of the protein and its sequence is required, tandem mass spectrometry (MS/MS) can be performed. During this procedure, the peptide fragments are captured as they fly through the instrument and further fragmented through collisional activation. In much the same way as described above for the MALDI QqTOF, during MS/MS the time of flight analyzer detects the length of time it takes these smaller peptide fragments to travel a certain distance. In this case, the fragments may differ by as little as one or two amino acids and the computer programs associated with the instruments are able to integrate the information obtained in the form of m/z ratios, into an amino acid sequence. Both of these techniques are highly sensitive, accurate and fast ways to identify and characterize proteins.

1.9 Characterization of Reovirus Antibodies

As mentioned above, antibodies play an important role in both clinical and laboratory settings. Current methods for obtaining and purifying reovirus antibodies require tedious procedures, large amounts of purified protein and a long period of time. Novel methods to obtain and characterize large quantities of reovirus antibodies are necessary to

facilitate both the current levels of research and aid in further studies in the future. Mass spectrometry and immunoprecipitation coupled with monoclonal antibody production offers a new and highly sensitive method to identify antigen and indirectly characterize antibodies. With this method, the need for purified antigen is eliminated in the initial screening and characterization of the antibodies, as the specific antigen can be identified by immunoprecipitation and mass spectrometry later.

1.10 Goals of This Research

As mentioned previously, the reovirus core particle is the structural form of the virus involved in the transcription of the viral RNA. However, little is known in regards to the protein interactions required for transcription to occur. While it has been demonstrated that the λ3 protein is the RdRp, the function of the μ2 protein as the RdRp cofactor remains speculative. In an effort to gain more information about both the ARV and MRV μ2 proteins, that might potentially better delineate the role(s) of these proteins in the viral replication cycle, nucleotide sequence determinations of the avian reovirus M1 genome segments of the ARV strains 138 and 176 (ARV 138 and ARV 176) were performed. Sequencing had not yet been undertaken for the other two avian reovirus M genes and in order to complete this, the M2 and M3 genes were sequenced as well. The ARV μ proteins were also purified and their predicted sequences were confirmed by mass spectrometry. After the sequences were obtained, I compared both the gene sequences and the ARV μA, μB and μNS with the MRV predicted equivalent proteins (μ2, μ1 and μNS, respectively) to identify conserved regions which may play a functional or structural role in the proteins and, with respect to the μ2/μA protein potentially be involved in transcription.

Another study was also undertaken to develop new tools for the study of cores and their transcriptional ability. In this study, dissociated cores were used to elicit antibody formation. Because dissociated cores were used, the mixture contained predominantly the major core proteins, λ1 and σ2. The immunogenicity of each of the five core proteins; λ1, λ2, λ3, μ2, σ2, is unknown. Over one hundred antibodies were initially screened. Of the initial group of antibodies, eleven were randomly chosen with a mix of Western Blot positive and negative antibodies. Limiting dilution was performed to ensure the antibodies were monoclonal and the antibodies were then screened and characterized by ELISA and Western Blot using the same type of dissociated cores used to elicit the antibody response in the host mouse. To identify antibody specificity, a modified immunoprecipitation procedure was performed. The antibodies of interest were captured by a primary antibody linked to sepharose beads and incubated with dissociated cores so the antibody could capture its specific antigen. This specific protein was then eluted from the monoclonal antibody of interest, digested with trypsin and analyzed by mass spectrometry in order to identify the protein. One monoclonal antibody was used in this preliminary study in which the reovirus-specific protocol was developed and optimized. The specificity of two of the original eleven monoclonal antibodies was determined by Western Blot.

2. Materials and Methods

2.1 Cells and viruses

Mouse fibroblast L929 cells were used for passage of mammalian reovirus. The cells were grown in suspension in Joklik modified minimal essential medium (MEM medium, Gibco BRL, Carlsbad, CA) containing 2.5% fetal calf serum (FCS) (Gibco BRL), 2.5 % VSP neonate bovine serum (Biocell, Rancho Dominguez, CA) and 2mM l-glutamine in the presence of 5% CO_2 at 37°C. They were counted and diluted 5 days each week to $5x10^5$ cells/mL. The cells were counted and diluted to $2.5x10^5$ cells/mL over the weekend. The medium was supplemented with 1X penicillin-streptomycin sulfate (100U/mL penicillin (Sigma, Oakville, ON), 100μg/mL streptomycin sulfate (Sigma) and 1X amphotericin B (1μg/mL) (Sigma) when cell monolayers were used for infection (Coombs *et al.*, 1994).

QM5 continuous quail cells were used for passage of avian reovirus. Cells were grown in monolayers in 1X Medium 199 containing 7.5% FCS (Gibco BRL), and 2 mM l-glutamine in the presence of 5% CO_2 at 37°C. Confluent cell monolayers were passaged twice weekly by standard trypsinization procedure and split 1:6. Media is removed, cells are washed with PBS/EDTA (137 mM NaCl, 0.3 mM KCl, 0.8 mM Na_2HPO_4, 0.1 mM KH_2P0_4, 0.05 mM EDTA) and then 0.25% trypsin (Sigma) is added. Cells were rocked until the monolayer detached and were then resuspended in QM5 maintenance media. QM5 maintenance media was supplemented with 1X penicillin-streptomycin sulfate (100U/mL penicillin, 100μg/mL streptomycin sulfate) and 1X amphotericin B (1μg/mL) when cells were used for infection (O'Hara *et al.*, 2001; Patrick *et al.*, 2001).

Reoviruses were from prototypic lab stocks of mammalian reovirus serotype Type 1 Lang (T1L) and avian reovirus serotypes Avian Reovirus 138 (ARV 138) and Avian Reovirus 176 (ARV 176).

2.2 Antibodies

The mouse monoclonal reovirus protein-specific antibodies were provided by Dr. John Wilkins (University of Manitoba). Monoclonal antibodies were produced by the fusion method, as previously described (Goding, 1986). A diagrammatic representation of the procedure outline can be found in Figure 4. Two female BALB/c mice were immunized subcutaneously three times with 100 μg of dissociated reovirus cores mixed with a synthetic adjuvant, Titermax Gold (CytRX, Los Angeles, CA). Four days before the harvesting of the spleen, each mouse was immunized intraperitoneally with 100 μg of dissociated reovirus cores without adjuvant. Each mouse was sacrificed and their spleens were removed, ground and the cells were collected. The cells were then washed to remove the media before the fusion. Fusion of the mouse spleen cells with SP2/0 myeloma cells was mediated by the slow addition of 50% polyethylene glycol (Sigma). HAT-containing media (hypoxanthine, aminopterin and thymidine) (Sigma) was added to the cells to select for the fused cells; they were then plated in 96-well plates and placed at 37°C, 5%CO_2 for approximately 12-14 days. Supernatant from each hybridoma was tested by ELISA. Positive cells were cloned twice by the limiting dilution method (Harlow, 1999) and tested by ELISA. Positive clones were allowed to grow to high numbers, cells in log phase were frozen down. Cells were grown to $5x10^5$ cells/mL in RPMI-1640 (Invitrogen) containing 10% FCS until death. The cells were then pelleted and the supernatant was collected (spent media). The antibodies were isotyped

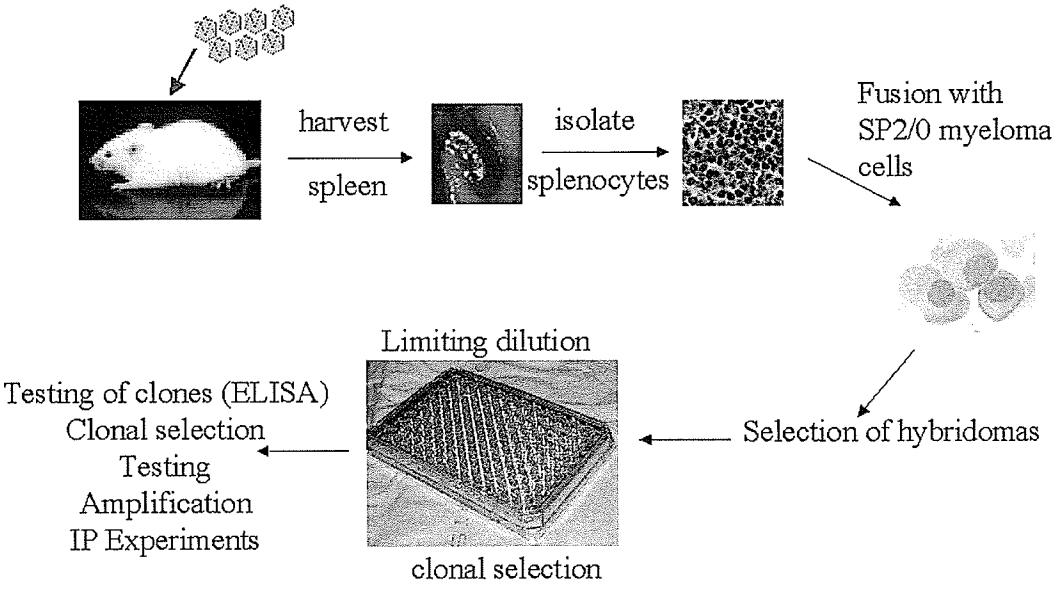

harvest
spleen
isolate
splenocytes
Fusion with
SP2/0 myeloma
cells
Selection of hybridomas
Limiting dilution
clonal selection
Testing of clones (ELISA)
Clonal selection
Testing
Amplification
IP Experiments

Figure 4. General Overview of the Production of Monoclonal Antibodies. In order to produce monoclonal antibodies, the antigen of interest is injected repeatedly into a mouse whose immune system synthesizes antibodies against the antigen (upper right). After several antigen injections, the mouse is sacrificed and its spleen is harvested. The spleen cells are extracted and fused with myeloma cells (centre right). Those cells which have fused and are producing antibody are selected out, tested by ELISA and amplified. The specificity of the antibody can then be confirmed using an ELISA (lower left). The antibody can then be used for experimental purposes.

using the Isotyping Monoclonal Antibodies Kit (Amersham). Spent media was used as a source of antibodies for ELISAs (section 2.6.1) and initial screening by Western Blot (section 2.6.2). In the case of #2-5C2, this spent media was used in the Western Blot final characterization for the antibody (section 2.6.2). Antibody #2-3C11 and #1-3C11 were purified using a HiTrap® protein G column (Amersham, Piscataway, NJ), as per the manufacturer's instructions. Briefly, the column was washed with 10 volumes of 20 mM sodium phosphate, pH 7.0, 50 mL of spent media was cycled through the column three times, the column was washed with five more volumes of 20 mM sodium phosphate, pH 7.0 and then the purified antibody was eluted from the column with 0.1 M glycine, pH 2.7 as 1 mL fractions. The fractions containing antibody were identified by OD_{280} readings, the fractions were pooled and the antibody was dialyzed against 2X phosphate buffered saline (PBS) (274 mM NaCl, 0.6 mM KCl, 1.6 mM Na_2HPO_4, 0.2 mM K_2HPO_4). The purified #2-5C2 was used in the final characterization Western Blot (section 2.62) and the purified #1-3C11 was coupled to cyanogen-activated sepharose beads (section 2.6.4) or A/G beads for an immunoprecipitation experiment (section 2.6.6).

2.3 Virus Manipulation

2.3.1 Viral Passage

L929 cells were used to amplify mammalian reovirus stocks and QM5 cells were used to amplify avian reovirus stocks. Tissue culture flasks were seeded with 4.5 x 10^5/mL cells and cells were grown to a sub-confluent monolayer. The overlaying media was removed and cells were infected with virus, at a multiplicity of infection (MOI) of 0.5 plaque forming units (pfu) per L929 cell or QM5 cell for 1 hour at room temperature

with periodic rocking. After 1 hour, a mixture of 25% preadapted media, 75% fresh media containing 100U/mL penicillin, 100μg/mL streptomycin sulfate and 1X amphotericin B (1μg/mL) was used to overlay the cells. The infection was then incubated at 37°C, in 5% CO_2, until an approximately 90% cytopathic effect was observed. Cells were lysed by 3 freeze/thawing cycles at -80°C, collected, titred by plaque assay and stored at 4°C.

2.3.2 Virus Plaque assay

Avian and mammalian reovirus stocks were serially diluted using gel saline (137 mM NaCl, 0.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 19 mM HBO_3, 0.1 mM $Na_2B_4O_7$, 0.3% [wt/vol] gelatin). Tissue culture 6-well plates were seeded and grown to sub-confluent monolayers and the overlaying media was removed. Cells were infected with the serially diluted virus and adsorption of the virus was allowed for 1 hour at room temperature with periodic rocking. After the adsorption period, L929 and QM5 cells were overlaid with a 1:1 mixture (v/v) of 2X Medium 199 (Gibco BRL), FCS, L-glutamine and sodium bicarbonate with 2% BactoAgar (Difco, Franklin Lakes, NJ), supplemented with 1X amphotericin B and 1X penicillin-streptomycin sulfate. Plates were incubated at 37°C, in 5% CO_2, for the appropriate length of time. Mammalian reovirus plaque assays were fed on day 4 post infection (p.i.) with the same overlay as described above, QM5 cells were fed on day 3 p.i.; both were stained when viral plaques became visible to the naked eye (usually day 7 p.i.). Cells were stained with 0.04% neutral red solution (Fisher, Nepean, ON) in a 1:1 mixture (v/v) of 2% Bacto agar and 2X phosphate-buffered saline (PBS) (274 mM NaCl, 0.6 mM KCl, 1.6 mM Na_2HPO_4, 0.2 mM K_2HPO_4). The next day, the plaques were counted and the titre of the virus was determined.

2.3.3 Mammalian Virus Purification

Reovirus serotype T1L was plaque purified and grown in mouse L929 cell monolayers in Joklik MEM containing 2.5% FCS, 2.5 % VSP neonate bovine serum, 2mM l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate and 1 μg/mL amphotericin B, as previously described (Coombs *et al*, 1994). Large amounts of virus were grown in spinner culture. Suspension cultures containing 6.5 x 10^8 L929 cells/ L were infected with T1L virus, at an MOI of 5 pfu per cell. The cells were pelleted, resuspended in virus and the virus was allowed to adsorb for 1 hour at room temperature, with periodic rocking, before fresh MEM was added. The infection was allowed to proceed for 65 hours at 33°C. The cells were then pelleted in a Beckman J2-HS centrifuge (4500 rpm, 45 mins.) and then resuspended in HO buffer (10 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.067% β-mercaptoethanol [v/v]). Cell lysis was carried out by the addition of 0.2% sodium deoxycholate (DOC) [w/v] and sonication (Mendez *et al*, 2000). Virus was then extracted with Vertrel-XF (a freon substitute manufactured by Dupont, Wilmington, DE) and purified in a 1.2-1.4g/cc cesium chloride gradient as previously described (Mendez *et al*, 2000). Purified virus bands were harvested and dialyzed against dialysis buffer (D buffer: 150 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris, pH 7.4).

2.3.4. Purification of ARV138

Avian Reovirus serotype 138 was plaque purified and grown in QM5 continuous quail cell monolayers in 1x Medium 199 containing 10% FCS, 10% tryptose phosphate broth, 2 mM l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate and 1 μg/mL amphotericin B. Large amounts of virus were also grown in cell monolayers.

Typically, 25 monolayer cultures of 2.79 x 10^7 QM5 cells in P150 dishes were infected with an MOI of 0.1 pfu per QM5 cell. The virus was allowed to adsorb for 1 hour at room temperature with periodic rocking, fresh media was added and the infection was allowed to proceed until approximately 40% cytopathic effect was visible. The cells were then harvested and pelleted in a Beckman J2-HS centrifuge. The pellet was resuspended in HO buffer and cell lysis was performed by the addition of 0.2% DOC and sonication, as described above for the mammalian virus T1L. The virus was extracted with Vertrel-XF and purified in a 1.2 g/cc cesium chloride- 1.32g/cc iodixanol (Amersham) gradient. Purified virus bands were harvested and dialyzed against D buffer.

2.3.5. Preparation of Mammalian Reovirus Cores

Purified virus was concentrated to 6.5 x 10^{13} particles/mL and α-chymotrypsin was added to a concentration of 200 μg/mL. The digestion was incubated at 37°C for 3 hours with vortexing every hour. Cores were then purified in 1.2-1.5 g/cc cesium chloride gradients as previously described (Yin *et al.*, 1996). Purified cores were harvested and dialyzed against core dialysis buffer (1M NaCl, 25mM HEPES, pH 8.0, 100 mM $MgCl_2$). Cores were dissociated with 9.0 M urea, dialyzed against core buffer overnight and dissociation was checked by electron microscopy.

2.4 Sequencing the ARV 138 and ARV 176 M1, M2 and M3 genes

2.4.1 Preparation of viral template

ARV 138 or ARV 176 was used to infect 4 P100 dishes of sub-confluent QM5 monolayers. The infection was stopped when approximately 80% cytopathic effect was observed. Cell debris, organelles and nuclei were pelleted by centrifugation, genomic dsRNA was then extracted with phenol/chloroform and the aqueous phase was

precipitated overnight at -20°C using $1/10^{th}$ the phase volume sodium acetate and 2.5X the phase volume ice-cold ethanol (Sambrook *et al.,* 1989). The precipitated RNA was spun down, washed, dried and then resuspended. For the internal sequencing reactions the RNA was resuspended in 90% dimethyl sulfoxide (DMSO)/10% DEPC-H_2O (diethyl pyrocarbonate) and for the 3' ligation reactions the RNA was resuspended in 1X PBS. The identity of each strain was confirmed by resolving aliquots of each virus in 10% SDS-PAGE and comparing band mobilities (Duncan, 1999).

2.4.2 Primer Design

Oligonucleotide primers corresponding to the 5' forward sense end and 5' reverse sense end of the ARV 138 M genes and additional oligonucleotides for sequencing were designed as needed and obtained from Invitrogen. 5' and 3' primers were created using a preliminary sequence of the ARV 138 M1 and M3 genes obtained from Dr. Roy Duncan (Dalhousie University, Halifax) (Table 2). Primers used in the 3' ligation reactions were lab primers designed by Dr. Paul Hazelton (University of Manitoba, Winnipeg) for another project. Internal primers for the 3' ligation sequencing of the ARV M2 genes were primers designed from the sequence of the ARV 138 M2 genes obtained from Dr. Roy Duncan (Dalhousie University, Halifax) (Table 2).

2.4.3 Reverse transcription polymerase chain reaction (RT-PCR)

cDNA copies of the ARV138 and ARV 176 M1 and M3 genes were constructed by RT-PCR. Purified dsRNA was denatured by heating at 50°C for 45 minutes and snap-cooled by placing the tube immediately in ice and adding ice cold DEPC- H_2O. RNase inhibitor (0.8 μL, 40 U/ μL) (Invitrogen, Carlsbad, CA), a mix of 10 mM each of dideoxynucleotides (dATP, dGTP, dCTP, dTTP) (5 μL), 10 μM oligonucleotide primers

Table 1- Avian Reovirus Primers for Amplification and Sequencing of the M Genes

Primer Name	Sequence	Orientation (wrt ORF)	Location
ARVM301	GCTTTTTGAGTCCTAGCGTGGATC	forward	1-24
ARVM302	CACCGCCAAGATCTTAGCTAA	forward	501-521
ARVM303	TGTCTACTCGGAATATTCGTGTC	forward	1001-1023
ARVM304	AAAGATCATGAAAAAGGTCTGC	forward	1501-1522
ARVM305	ACGAGCGTGATGAATTACTC	forward	1730-1750
ARVM306	GATGAATAACCGAGTCCGCCGTG	reverse	1996-1973
ARVM307	GTTCAAGTATTGTAGGGAATTGA	reverse	1722-1701
ARVM308	CTTGGCAATTTGTTCTTTTAGCT	reverse	1323-1301
ARVM309	TAAGACGTGTAGAAAAGATAGTCAA	reverse	775-751
ARVM310	CAAGCATCTCAACAGCCTGA	reverse	279-260
ARVM311	CTGATGCAGTAAGACGTGTGAA	reverse	275-256
ARVM312	CTTATCGGTTCCTCTGTCTCACTC	forward	112-135
ARVM313	AGGGTTGAGTCATCATGTCCTG	reverse	1111-1089
ARVM101	GCTTTTCTCGACATGGCCTATC	forward	1-22
ARVM102	TTCTGTCGCCGGACGTTC	forward	504-521
ARVM103	GGGTGCCCATTCGTGATCA	forward	1002-1030
ARVM104	GCCTTAGAATTATACTCGGAGC	forward	1511-1532
ARVM105	ATTCGTGCGGTTAAGGTGTC	forward	2021-2039
ARVM106	GATGAGTATCTCAAGACGACTAACC	reverse	2281-2257
ARVM107	GGCACAAACAATGCATTGGGAA	reverse	1773-1752
ARVM108	TCCAGTAAGAAATTTAATCATGG	reverse	1273-1251
ARVM109	CCGACCGATTTCCAAGAAGG	reverse	770-751
ARVM110	ATAGATATTCAAGGAGAGAACGAC	reverse	275-252
ARVM111	TTGTCTAATCCTTCCGCTTGC	forward	280-300
ARVM112	TGTATGATCACGAATGGGCAC	reverse	1030-1010
3'L1	CCCCAACCCACTTTTTCCATTACGCCCCTTTCCCCC	reverse	(a)
3'L2	GGGGGAAAGGGGCGTAATGGAAAAAGTGGGTTGGGG	forward	(a)
3'L3	GGGGGAAAGGGGCGTAAT	reverse	(a)
ARV138M25'END	ACGTCTCTTTAGTGGCGATG	reverse	327-308
ARV138M23'END	GCCTCACGAGCTCTTCACAT	forward	1818-1837
ARV176M25'END	TGCATGCTCAGTTATTACACTTA	reverse	327-308
ARV176M23'END	GCTCTTCACATGTTCTTGGGT	forward	1818-1837

(a) 3'L1, 3'L2 and 3'L3 primers were ligated onto the tail end of each gene and were located outside the open reading frame (ORF)

(1 μL), 5X 1st strand buffer (Invitrogen), 10 mg/mL bovine serum albumin (BSA) (1 μL), 100 mM dithiothreitol (DTT) (1 μL) and Superscript II reverse transcriptase (0.8 μL, 200 U/ μL) (Invitrogen) were then added and the mixture was incubated at 42°C for 2 hours to produce cDNA. The cDNAs were amplified by polymerase chain reaction (Saiki *et al.,* 1985) using the Expand Long Enzyme System (Roche, Laval, PQ) and the Expand Long Buffer 1, containing 17.5 mM $MgCl_2$. The 30 cycling reactions were performed using the PTC-100, Programmable Thermocycler (MJ Research Inc., Waltham, MA). PCR consisted of an initial denaturation of the dsDNA at 96°C for 2 mins, 30 amplification cycles of 92°C for 1 min (denaturation of the strands), 53°C for 1 min 30 secs (primer annealing), 68°C for 2 mins 45 secs (DNA synthesis), a final 68°C for 10 mins to complete DNA synthesis and a hold at 4°C to maintain the integrity of the newly-formed PCR products. PCR products were resolved in 1% agarose gels stained with ethidium bromide (Sambrook *et al.,* 1989), and the bands corresponding to the genes of interest were excised, purified and eluted with Qiagen® columns, pursuant to the manufacturer's instructions (Qiagen Inc., Mississauga, Ontario). Sequences of these cDNAs were determined with gene-specific internal oligonucleotides by dideoxy-fluorescence methods.

2.4.4 3' Ligation for ARV Gene-End Sequencing

The sequences at the ends of all genes were determined in both directions by a modification of the 3' ligation method described by Lambden *et al* (1992). ARV genes were extracted by phenol-chloroform as described above. Genes were then resolved in a 1% agarose (Roche) gel and the M genes of both ARV 138 and ARV 176 were excised and eluted as described above with Qiagen® columns, using the manufacturer's

instructions. Oligonucleotide 3'L1 (Table 2) (phosphorylated at the 5' end and blocked with a biotin group at the 3' end) was ligated to the 3' ends of the M genes. The Qiagen® purified gene was mixed together, on ice, with primer 3'L1, T4 RNA ligase buffer and T4 RNA ligase (Roche), and incubated at 37°C overnight. The ligated genes were repurified by agarose gel and Qiagen® columns to remove unincorporated 3'L1 oligonucleotide and precipitated overnight with sodium acetate and ice-cold ethanol at -20°C. The precipitated genes were washed with ethanol, dried and dissolved in 6µl of 90% DMSO. cDNA copies of the ligated M genes were constructed by using oligonucleotide 3'L2 (Table 2) and gene-specific internal oligonucleotide primers (ARV M101/ARVM106, ARV 138 M2 5'/ARV 138 M2 3', ARV 176 M2 5'/ARV 176 M2 3'or ARVM301/ARVM306) designed to generate a product of approximately 0.5 kb in length. These constructs were amplified by PCR; purified in 1% agarose gels, excised, and eluted as described above. Sequences of these cDNAs were determined with gene-specific internal oligonucleotides and with oligonucleotide 3'L3 (Table 2) by dideoxy-fluorescence methods.

2.4.5 Cycle sequencing reactions

Sequences of the respective cDNAs were determined in both directions by dideoxynucleotide cycle sequencing (Sanger *et al.*, 1977; Murray, V., 1989; Craxton, 1991), using fluorescent dideoxynucleotides. The purified PCR products were mixed, on ice, with Big Dye (Applied Biosystems, Foster City, CA), 1µM primer and ddH_20. Cycle sequencing was performed with a GeneAmp PCR System 9700 (Applied Biosystems) with the following conditions: 94°C for mins, 94°C for 1 min, 52°C for 1 min, 70°C for 2 min, cycle back to 94°C for 1 min 34 subsequent rotations, 7°C for 7 mins and hold at

4°C. The cycle sequencing products were then precipitated in the dark for several hours using sodium acetate and ice-cold ethanol. Each sample was then pelleted, washed and dried completely. Ultra-pure formamide was added to each tube, the sample was heated to 90°C, snap-cooled in wet ice and then transferred to a 96-well sequencing plate. Fluorescence dideoxysequencing was performed using an ABI3100 Sequence (Applied Biosystems).

2.4.6 Sequence Analysis

Sequences were initially analyzed with the DNASTAR® suite (Madison, WI). Each gene was sequenced in both directions and contigs were generated by inputting each direction into the DNASTAR® Seqman program. After the gene sequence from each ARV strain was confirmed, both ARV 138 and ARV 176 gene sequences were aligned and confirmed again. The DNASTAR® EditSeq program was then used to translate each gene. ExPasy (Geneva, Switzerland), Network Protein Service (Lyon, France), PredictProtein (Heidelberg, Germany) and BLAST (Bethesda, MD) programs were used for protein sequence analysis. Multiple sequence alignments were performed using BoxShade (EMBnet, Switzerland), phylogenetic analysis was performed using GeneBee (Moscow, Russia) and pairwise comparisons of both gene and protein sequences were done using Lalign (EMBnet, Switzerland). The default settings were used for all analysis.

2.5 Proteomic Analysis of Avian Reovirus Proteins

2.5.1 Protein Assay

Viral protein was quantified by standard protein assay. A bovine serum albumin (BSA) (Bio-Rad, Hercules, CA) standard curve was prepared using 6 serial dilutions of BSA. Unknown protein samples were also serially diluted and each of the unknowns and

standard samples was then loaded onto an ELISA plate. Bicinchoninic acid (BCA) solution (Pierce, Rockford, IL) was prepared, added to each well and the plate was incubated at 37°C for 30 minutes. The plate was then read on an ELISA plate reader at 550 nm, the blank OD was subtracted from the other OD readings and the protein concentration was calculated.

2.5.2 Tris-Glycine-Urea SDS-PAGE of Avian Reovirus Proteins

Standard SDS-PAGE does not sufficiently separate the reovirus μ proteins for mass spectrometry so alternate methods were required. Tris-Glycine-Urea (TGU) gels have been used to thoroughly resolve mammalian reovirus proteins (Coombs, 1998) and were undertaken here in an attempt to separate the avian reovirus proteins. The TGU gel was 16 cm x 13.5 cm x 0.1 cm and consisted of a step of 4% acrylamide gel, layered on top of a linear 4-4.5% acrylamide gel, which was on top of a 4.5-16% acrylamide exponential gradient. The gel was allowed to polymerize for 8 hours. A 3.75% standard stacking gel was then poured and allowed to polymerize. Each avian reovirus sample (30 μg of viral protein) was mixed with 5X-electrophoresis sample buffer [25% 1.25 M Tris, pH 6.8, 10% SDS (w/v), 0.01% Bromophenol Blue (Sigma), 50% glycerol, 8% DTT(w/v)] and heated at 95°C for 5 minutes before loading. Once the samples were loaded, the gel was run at 9 mA for approximately 14 hours, 1 hour after the dye front had run off the bottom of the gel. Following the run, the gel was removed from the gel-running apparatus, fixed for at least 30 minutes in a fixative solution (25% methanol, 10% acetic acid) and stained overnight using GelChip Blue Colloidal Coomassie Blue Stain (Proteome Systems Ltd., Woburn, MA). Spots were cut out of the gel and subjected to in-gel digestion and mass spectrometry. Gel pieces were stored for short periods of

time (days up to a week) covered in 1% acetic acid or, for periods longer than a week, they were stored dry at -80°C.

2.5.3 In-Gel Digestion

Coomassie Blue stained gel pieces were first washed with ammonium bicarbonate (NH_4HCO_3) to neutralize the stain. Each piece was then cut into tiny pieces and washed with 30-40% acetonitrile (ACN) in 100 mM NH_4HCO_3 to remove the dye; this step involves the shrinkage of the gel. This liquid was then removed and the gel pieces were covered with ACN and incubated for 5 minutes to swell the gel piece. This ACN/ NH_4HCO_3 incubation followed by ACN incubation procedure was continued until all the dye had been removed from each gel piece. After the last wash, the gel piece was vacuum dried, swollen with 100 mM DTT and incubated at 57.5°C for 45 minutes. This step was required for the reduction of the protein in the gel spot. After the reduction, the DTT was removed, 500 mM iodoacetamide was added and the sample was incubated in the dark for 30 minutes in order to alkylate the proteins. The gel piece was then washed thoroughly and ground up in digestion buffer (5 ng/μL trypsin in 50 mM NH_4HCO_3) and incubated overnight at 37°C. The liquid was collected and the trypsin digestion was stopped with 0.01% Trifluoroacetic acid (TFA). The peptides were extracted from the gel with 0.001% TFA in 50% ACN and dried. The peptides were then resuspended in 10 μL of 0.1% TFA and purified using a ZipTip (Millipore, Billerica, MA). Briefly, the ZipTip was equilibrated with 50% ACN and 0.1% TFA. The peptides were then bound to the column by 10 cycles of aspiration and dispensation and the column was washed with 2 cycles of 0.1% TFA to clean away any unbound protein. The peptides were eluted from the column with 10 μL of 50% ACN in 0.1% TFA and used for mass spectrometry.

Another method used for in-gel digestion was adapted from Terry *et al*, 2004. In this method, gel pieces were excised, cut into pieces approximately 1 mm^3 and not destained. The pieces were washed twice with 50% ACN: 50% 200 mM NH_4HCO_3, dehydrated with 100% ACN until the gel was opaque and then dried in a vacuum centrifuge. Each gel piece was rehydrated with 50 mM NH_4HCO_3 at 37°C and then an equivalent volume of 8.35 ng/μL trypsin (Promega) was added. The digestion was allowed to proceed for 1 hour at 37°C. The peptides were extracted 3 times with 60% ACN in 5% TFA by sonication and the pooled supernatants were dried in a vacuum centrifuge. The peptides were resuspended in 10 μL of 0.1% TFA and purified using a ZipTip, as described above.

2.5.4 Mass spectrometric analysis

The dried peptides extracted from the gel spots, mixed 1:1 (v/v) with 160 mg/mL 2,5-dihydroxybenzoic acid in acetone/water (MALDI matrix solution) were spotted onto a gold chip. Analysis of the peptide sample was performed by matrix assisted laser desorption ionization quadrupole time of flight mass spectrometry (MALDI QqTOF). Mass spectra were analyzed using M/Z and Knexus proteomics programs (Genomic Solutions, Boston, MA). Average monoisotopic masses of the ionized peptide fragments were searched with ProFound against the NCBInr virus database (http://hs1.proteome.ca/prowl/knexus.html) as well as the unpublished avian reovirus sequences discussed in section 2.4.6. Selected peptide ions were further fragmented and analysed by tandem mass spectrometry (MS/MS) and data were analyzed by the Knexus suite of proteomics programs.

2.6 Characterization of Reovirus Antibodies

2.6.1 ELISA

In order to identify positive monoclonal antibodies, as well as ensure that the antibodies were able to recognize the dissociated reovirus core antigen, enzyme-linked immunosorbent assays (ELISAs) were performed. Maxisorb 96 well plates (Nunc, Rochester, NY) were coated with 5-10 μg/mL of dissociated reovirus cores and incubated at 4°C overnight. The plates were then washed with PBS+0.05% Tween 20 (Fisher) and blocked for 1.5 hours with PBS+1% BSA at room temperature. The plates were washed again, incubated with 100 μL of primary antibody supernatant and controls [normal mouse serum, immunized mouse serum, Chessie 6 (α-HIV gp120)-1: 500 dilution] for 1.5 hours at 37°C. After further washing, the plates were incubated at room temperature for 1 hour with the secondary antibody, a rabbit anti-mouse IgG alkaline phosphatase conjugate (Sigma), diluted 1:2000. The plates were then washed one final time and detected at room temperature with the alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma). Plate readings were performed at 405 nm after 15 minutes, 30 minutes and 1 hour.

2.6.2 Western Blots

Dissociated core proteins (approximately 200 μg/gel) were resolved in 8.5 x 6.7 cm x 0.75 mm 10% SDS-PAGE gel (Laemmli, 1970) using a preparative comb (BioRad). Reovirus core λ proteins were also resolved in a 6.0 x 8.5 cm x 0.75 mm 10% SDS-PAGE gel. The gels were then placed in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol) for approximately 30 minutes. The protein bands were transferred onto an Immobilon-P membrane (Millipore, Billerica, MA) by a semi-dry

method for 2 hours. The membrane was then blocked overnight at 4°C in Blocking Buffer (1% BSA, 0.02% Tween 20 in TBST). Primary antibody was applied to the membrane using the Mini-Protean II Multi-Screen Apparatus (BioRad). The apparatus allows the processing of 16 antibodies and 1 control per gel. Western Blots using only one antibody did not require the use of the slot blot apparatus; these Blots were performed in a conical centrifuge tube. The primary reovirus antibody supernatants were diluted 1:1 in blocking buffer, applied to the membrane, and incubated at room temperature for 2 hours. Purified antibody was diluted to 5 μg/mL. The membrane was then washed, removed from the Multi-Screen Apparatus or centrifuge tube, washed further and then incubated for 1 hour in secondary antibody, rabbit anti-mouse alkaline-phosphatase conjugate (Sigma) diluted 1:3000. After the last incubation, the membrane was washed and placed in alkaline phosphatase substrate (Buffer C- 0.1 M Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, BCIP-5-bromo-4-chloro-3-indolyl phosphate and NBT-nitroblue tetrazolium) for detection.

2.6.3 Electro-Elution of Reovirus λ Proteins from TGU Gels

Reovirus λ proteins were separated by TGU gel electrophoresis (see section 2.5.2). Gels were fixed (25% methanol, 10% acetic acid), stained with Coomassie Blue (Sigma) and destained overnight with 15% methanol, 7.5% acetic acid. Bands were excised using fresh razor blades and cut into pieces approximately 1 mm^3. A BioRad Model 422 Electro-Eluter was used to elute the proteins from the gel. Briefly, the membrane caps of the electro-eluter were soaked in protein elution buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 1 hour at 60°C. The gel pieces were then loaded into the electro-eluter and covered in protein elution buffer. The proteins were eluted at 8

mA/glass tube overnight at 4°C. Following elution, the liquid containing the eluted protein was collected and a 10% mini SDS-PAGE gel was run to determine the success of the elution and approximate concentration of the protein.

2.6.4 Antibody Column Preparation

The antibody solution used in the antibody column preparation was a goat anti-mouse antibody (Chemicon International Inc., Temecula, CA). As well, purified α-reovirus core antibody #1-3C11 was also coupled to sepharose beads using this procedure. The concentration of antibody used for the preparation was 1-2 mg of antibody per mL of cyanogen bromide-activated sepharose 4B beads (Sigma). Antibody was thoroughly dialyzed in double distilled water for three hours before coupling was performed. Coupling buffer (0.1 M $NaHCO_3$, 0.5M NaCl) was first prepared and the pH was adjusted to between 8.32 and 8.45 using Buffer B (0.1M Na_2CO_3, 0.5M NaCl). Beads were washed with 1 mM hydrochloric acid 4 times and then activated with coupling buffer. After the coupling buffer was added to the beads, the dialyzed antibody solution was immediately added to the bead slurry and the slurry was incubated at room temperature for 2 hours on a rotator. After the coupling, the slurry was centrifuged, the supernatant was removed and the remaining wet beads were covered in 1M ethanolamine buffer to block the remaining active groups. The slurry was stored overnight at 4°C. The next morning, the coupled beads were washed in alternating cycles of coupling buffer and acetate buffer (0.1M NaOAc, 0.5M NaCl, pH 4.0). The final set of washes was with 1X PBS. The centrifuge tube containing the washed, coupled beads was filled with 1X PBS and 0.02% sodium azide for long term storage of the beads at 4°C. Successful coupling of the antibody to the beads was confirmed by SDS-PAGE.

2.6.5 Immunoprecipitation Linked to Mass Spectrometry

Reovirus core specific antibodies were characterized by a modified immunoprecipitation experiment (Weiler *et al,* 2003). Goat anti-mouse IgG antibodies coupled to sepharose beads (see section 2.6.4) (50μL) were packed into siliconized tubes and incubated for 1 hour with 1 mL (approx. 400 μg) of reovirus specific antibody. The IgG and reovirus antibodies were then washed and incubated for 1 hour with reovirus core particles that had been previously dissociated with high concentrations of urea (8.5 M). After the incubation, the beads were centrifuged and the supernatant was removed. The beads were then washed with three cycles of RIPA Buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris (pH 7.5), 0.5% DOC, DNase I, Protease inhibitors), High Salt PBS (1 M NaCl) + 0.1% Triton X-100, 1X PBS + 1% Triton and 1XPBS. Antigen was eluted from the beads by puncturing a hole in the bottom of the tube with a 30 gauge needle, eluting the antigen off the beads with 100 mM glycine-HCl (pH 3.0) into a Microcon YM-10 filter (Millipore). Ammonium bicarbonate (50 mM) was then added to the eluted antigen. The rest of the antigen was reduced with 100 mM DTT and then alkylated with 500 mM iodacetamide. Subsequently, the sample was digested with trypsin, at a concentration of 5 ng/μL, overnight, dried and resuspended in Buffer A (95% H_20, 5% acetonitrile, 0.1% formic acid, 0.005% heptafluorobutyric acid). The sample was then analyzed by liquid chromatography (C_{18} column)-linked to tandem mass spectrometry (MS/MS) to determine the identity of the antigen and the specificity of the antibody. A small number of samples were resuspended in 50 mM NH_4HCO_3 and analyzed by MALDI QqTOF to determine the identity of the antigen and specificity of the antibody.

2.6.6 Immunoprecipitation Linked to SDS-PAGE

Reovirus core specific antibodies were further characterized by immunoprecipitation linked with SDS-PAGE and MALDI QqTOF. Immunopure immobilized protein A/G beads (40 μL) (Pierce) were incubated for 2 hours at 37°C with reovirus core specific monoclonal antibody #1-3C11 (0.5 mg). The supernatant was removed and the beads were washed 3 times with Immunopure Ig A/G binding buffer (Pierce). Dissociated reovirus cores (400 μg) were then incubated with the beads and antibody for 1 hour at room temperature. Beads that had #1-3C11 directly coupled to them were also used in this experiment (section 2.6.4) and incubated with the same amount of #1-3C11. The supernatant was removed and the beads were separated into 20 μL aliquots. The first aliquot was washed with 3 cycles of 100 mM β-octyl glucoside (BOG); 1% Triton X-100 in 1X PBS and 1X PBS and the other aliquot was washed with 0.05% DOC; 1% Triton X-100 in 1X PBS and 1X PBS. After washing, the beads/antibody/antigen complex was loaded onto a 10% linear mini-SDS PAGE gel, fixed, stained with Coomassie Blue and destained. Bands were excised and subjected to in-gel digestion (section 2.5.3) and mass spectrometry.

3. Results

3.1 ARV M Gene Sequence Analysis

Until recently, the only ARV genes that had been sequenced were the S genes. The M genes and the majority of the L genes had not yet been sequenced and this information was needed to better understand the ARV life cycle. As well, comparisons of the ARV and MRV S genes demonstrated similarities in the genes and the encoded proteins, suggesting similar structure and function. In this study, the initial focus was the M1 gene and its encoded product μA, a minor core protein that is the hypothesized RdRp cofactor. In order to identify regions within the MRV μ2 which may play essential structural and functional roles, the ARV M1 gene and its predicted protein, μA, were sequenced by traditional sequencing methods and analyzed to identify highly conserved regions. Because the other ARV M genes had also not yet been sequenced, traditional sequencing methods were used to sequence and analyze the ARV M2 and M3 genes and their predicted proteins, μB and μNS, respectively.

In order to sequence the individual ARV M genes, ARV 138 and ARV 176 were amplified and their dsRNA was extracted. RT-PCR was used to obtain copies of the ARV M1 and M3 genes and dideoxy-fluorescence methods were used to determine the sequence of each gene (section 2.4). Our collaborators in Halifax sequenced the ARV M2 genes and provided the sequence to us for gene-end sequencing and sequence analysis. Computer programs were used to identify the ORF of each gene and corresponding predicted protein. Each predicted protein was then analyzed further to determine expected characteristics, *i.e.* secondary structure, isoelectric point, hydropathy, buried residues, motif identification, BLAST searches and alignments.

3.1.1 M1 gene and μA protein sequence of ARV138 and ARV176

The nucleotide sequences of the M1 genome segments of avian reovirus ARV138 (GenBank Number AY557188) and ARV176 (GenBank Number AY557189) were determined. As seen in Table 3, both ARV M1 genes were 2283 base pairs long, with a 5' non-translated region of 12 bases and a 3' non-translated region of 72 bases. This is shorter than the M1 segments of the prototype members of the reovirus family, the MRV reoviruses T1L, T2J and T3D that have 2304 nucleotides, 2303 nucleotides and 2304 nucleotides, respectively (Wiener *et al.,* 1989; Zou and Brown, 1992; Yin *et al.,* 2004). The ARV138 M1 gene shares 91.8% homology (188 mismatches) with the ARV176 M1 gene (Table 4). Alignment of the 5' and 3' non-translated regions of ARV138 and ARV176 reveals complete sequence identity in these regions (Figure 5A). The non-translated regions of M1 from both ARV138 and ARV176 contain the complete conserved 5'- GCTTT and TCATC-3' sequences seen in all other sequenced ARV genes, indicating that the clones contain full length sequences. Like the MRV sequences, the ARV138 and ARV176 M1 plus-strand sequences contain a single long open reading frame with the same start and stop codons; however, the ARV open reading frame encodes a μA protein of 732 amino acids, while the MRV open reading frame encodes a μ2 protein of 736 amino acids.

3.1.2 M2 gene and μB protein sequence of ARV138 and ARV176

The nucleotide sequences of the M2 genome segments of avian reovirus ARV138 (GenBank Number AY750052) and ARV176 (GenBank Number AY750053) were determined, in collaboration with Dr. Roy Duncan. As seen in Table 3, both ARV M2 genes were 2158 base pairs long, with a 5' non-translated region of 29 bases and a

Table 3-Features of ARV 138 and ARV 176 M Genes and μ Proteins

	138 μA	176μA	138 μB	176 μB	138 μNS	176μNS
total nucleotides	2283	2283	2158	2158	1996	1996
5' NTR	12	12	29	29	24	24
3' NTR	72	72	98	98	64	64
total amino acids	732	732	676	676	635	635
mass (kDa)	82	82.2	73.1	73.3	70.9	70.8
pI	8.4	8.42	5.24	5.53	5.92	5.88
Asp and Glu	69	69	69	68	85	84
Arg and Lys	74	74	56	58	73	72
secondary structure	alpha-beta	alpha-beta	alpha-beta	alpha-beta	mixed	mixed

Table 4- Pairwise Comparisons of ARV 138 and ARV 176 µA proteins with Mammalian Reovirus µ2 proteins

	Identity (%) compared with reovirus isolate [a]				
Isolate	138 µB	176 µB	T1L µ1	T2J µ1	T3D µ1
138µB	·	95.3	45.3	45.3	45.1
176 µB	**90.4**	——	45.2	45	45
T1L µ1	**55.2**	**55**	——	97.5	98.2
T2J µ1	**54.8**	**55.9**	**76.9**	——	97.2
T3D µ1	**54.8**	**55.6**	**85.1**	**76.7**	——

[a]Abbreviations defined in text

[b]Genbank Nos. found in legend for figure 2,5 and 8.

[c]Values for M-gene sequence comparisons are shown below the diagonal, in bold; values for µ protein comparisons are shown above the diagonal

A)

138 M1 GCTTTTCTCGAC----
CGGGCCATGGGGCGGTGACACCCAGGGAGGGTATGCTGGTAACCCTGGGTTAGTCGTCTTGAGATACTCATC

176 M1 GCTTTTCTCGAC----
CGGGCCATGGGGCGGTGACACCCAGGGAGGGTATGCTGGTAACCCTGGGTTAGTCGTCTTGAGATACTCATC

B)

138 M2 GCTTTTTCAGTGCCAGTCTTTCTCACAAG----
GGTGCTAAGGCCTCTCTCTGCGGCGGGTCGGTGGGCACGTCGTGGTGACGCTGAATGCACGGGGAGGTGACGCTCCCTG
GATTGGCACGTTATTCATC

176 M2 GCTTTTTCAGTGCCAGTCTTTCTCACAAA----
GGTGCTAAGGCCTCTCTCTGCGGCGGGTCGGTGGGCACGTCGTGGTGACGCTGAATGCACGGGGAGGTGACGCTCCCTG
GATTGGCACGTTATTCATC

C)

138 M3 GCTTTTTGAGTCCTAGCGTGGATC----
GCTTTGACCTGTGATTCGGCTTCTCTCTGATTCCATGTGCTCACGGCGGACTCGGTTATTCATC

176 M3 GCTTTTTGAGTCCTAGCGTGGATC----
GCTTTGACCTGTGATTCGGCTTCTCTCTGATTCCATGTGCTCACGGCGGACTCGGTTATTCATC

Figure 5. Sequences of the 5' and 3'non-translated regions of ARV138 and ARV176. The non-translated regions of the M genes from both ARV138 and ARV176 contain the completed conserved 5'- GCTTT and TCATC-3' sequences seen in all other sequenced reovirus genes, indicating that the clones contain full length sequences. A) The 5' and 3' non-translated regions of the prototype avian reovirus M1 genes. ARV138 (avian reovirus 138, GenBank No. AY557188) and ARV176 (avian reovirus 176, GenBank No. AY557189). B) The 5' and 3' non-translated regions of the prototype avian reovirus M2 genes. ARV138 (avian reovirus 138, GenBank No. AY750052) and ARV176 (avian reovirus 176, GenBank No. AY750053). C) The 5' and 3' non-translated regions of the prototype avian reovirus M3 genes. ARV138 (avian reovirus 138, GenBank No. AY557190) and ARV176 (avian reovirus 176, GenBank No. AY557191).

3' non-translated region of 98 bases. This is shorter than the M2 segments of the prototype members of the reovirus family, the MRV reoviruses T1L, T2J and T3D that have 2203, 2203, and 2207 nucleotides, respectively (Chandran *et al.* 1999; Wiener and Joklik, 1988; Tarlow *et al.,* 1988). The ARV138 M2 gene shares 90.4% homology (208 mismatches) with the ARV176 M2 gene (Table 5). Alignment of the 5' and 3' non-translated regions of ARV138 and ARV176 reveals complete sequence identity in these regions (Figure 5B). The non-translated regions of M2 from both ARV138 and ARV176 contain the complete conserved 5'- GCTTT and TCATC-3' sequences seen in all other sequenced ARV genes, indicating that the clones contain full length sequences. Like the MRV sequences, the ARV138 and ARV176 M2 plus-strand sequences contain a single long open reading frame with the same start and stop codons; however, the ARV open reading frame encodes a μB protein of 676 amino acids, while the MRV open reading frame encodes a μ1 protein of 708 amino acids.

3.1.3 M3 gene sequences and μNS sequence of ARV138 and ARV176

The nucleotide sequence of the M3 genome segment of ARV strains 138 (GenBank Number AY557190) and 176 (GenBank Number AY557191) were determined. Both genes were 1996 nucleotides long, with a 5' non-translated region of 24 bases and a 3' non-translated region of 64 bases, as seen in Table 3. Like the ARV M1 gene, this is shorter than the MRV M3 genes that are 2241 base pairs (T1L), 2240 base pairs (T2J) and 2241 base pairs (T3D) (McCutcheon *et al.,* 1999). The 5' and 3' non-translated regions are considerably longer in the ARV M3 than the MRV M3 and alignment of the two ARV non-translated regions show them to be identical (Figure 5C). The non-translated regions of M3 from both ARV138 and ARV176 contain the

Table 5- Pairwise Comparisons of ARV 138 and ARV 176 µB proteins with Mammalian Reovirus µ1 proteins

	Identity (%) compared with reovirus isolate [a]				
Isolate	138 µB	176µB	T1L µ1	T2J µ1	T3D µ1
138 µB	———	95.3	45.3	45.3	45.1
176 µB	**90.4**	———	45.2	45	45
T1L µ1	**55.2**	**55**		97.5	98.2
T2J µ1	**54.8**	**55.9**	**76.9**	———	97.2
T3D µ1	**54.8**	**55.6**	**85.1**	**76.7**	———

[a]Abbreviations defined in text

[b]Genbank Nos. found in legend for figure 2,5 and 8.

[c]Values for M-gene sequence comparisons are shown below the diagonal, in bold; values for µ protein comparisons are shown above the diagonal

complete conserved 5'- GCTTT and TCATC-3' sequences seen in all other sequenced ARV genes, indicating that the clones contain full length sequences. The ARV138 M3 gene shares 93.6% homology (110 mismatches) with the ARV176 M3 gene (Table 6). Like the MRV sequences, the ARV138 and ARV176 M3 plus-strand sequences contain a single long open reading frame with the same start and stop codons; however, the ARV open reading frame encodes a μNS protein of 635 amino acids, while the MRV open reading frame encodes a μNS protein of 719 amino acids.

3.2 ARV Predicted μ Protein Sequence Analysis

3.2.1 Comparisons of the μA protein sequences

As shown in Table 3, the encoded μA proteins are predicted to have molecular weights of 82.0 kDa (ARV138) and 82.2 kDa (ARV176). The predicted isoelectric points of the two ARV μA proteins are 8.40 (ARV138) and 8.42 (ARV176). These are much higher than the isoelectric points of the MRV μ2 proteins, which are 6.9, 7.44 and 7.0 for T1L, T2J and T3D, respectively (Yin *et al.,* 2004). This dramatic difference in isoelectric point between the MRV and ARV proteins suggests a high level of variation between them. The ARV μA proteins share 97.7% homology (17 substitutions out of 732 amino acids). The majority of the amino acid substitutions result in a conservative change to an amino acid of similar size and charge (data not shown). Alignment of the MRV μ2 protein and the ARV μA proteins showed that the two proteins share approximately 30% amino acid identity, predominantly in the conserved regions of the MRV μ2 protein designated CR7 (Figure 6) (Yin *et al.*, 2004). Conserved regions 5 and 6 contain sequence bearing strong resemblance to the A and B motifs of known ATPases (Noble

Table 6- Pairwise Comparisons of ARV 138 and ARV 176 µNS proteins with Mammalian Reovirus µNS proteins

	Identity (%) compared with reovirus isolate [a]				
Isolate	138 µNS	176 µNS	T1L µNS	T2J µNS	T3D µNS
138 µNS	——	97	25.2	27.2	26.8
176 µNS	**93.6** [c]	——	24.9	25.9	26.6
T1L µNS	**49.6**	**49.1**	——	82.4	94.5
T2J µNS	**48.6**	**48.9**	**72.1**	——	82.5
T3D µNS	**49.4**	**49.4**	**87.5**	**71.5**	——

[a]Abbreviations defined in text

[b]Genbank Nos. found in legend for figure 2,5 and 8.

[c]Values for M-gene sequence comparisons are shown below the diagonal, in bold; values for µ protein comparisons are shown above the diagonal

and Nibert, 1997) and a similar sequence is seen in the ARV μA proteins. Comparisons of the ARV μA protein and MRV μ2 protein region reveal amino acid substitutions resulting in amino acids of the same type and size; for example the replacement of a valine residue in the ARV μA (residue 685) with a lysine residue in the MRV μ2 (residue 695). The MRV μ2 protein is a putative RdRp cofactor and the regions of protein sequence similarity between the ARV and MRV proteins encoded by the M1 genes suggests the ARV μA protein may also be an RdRp cofactor with NTPase activity. BLAST analysis suggests lesser amino acid sequence identity with several aquareovirus proteins and alignment of the aquareovirus, MRV and ARV proteins demonstrates several conserved regions, including CR5 and CR6 (Figure 6). The phylogenetic analysis shown in Figure 7 demonstrates a closer phylogenetic relationship between the ARV and MRV reoviruses, than the ARV and aquareoviruses.

Predictions of μA secondary structure (using the Kyte-Doolittle regional hydropathy, ExPasy ProtScale surface probability and Network Protein Sequence Analysis) were also determined. The ARV138 and ARV176 hydropathy profiles, as well as surface probability profiles were identical and show numerous regions that are predicted to be exposed at the surface of the protein as well as several regions predicted to be buried (Figure 8A and 8B). The predicted secondary structure of the ARV μA protein is shown in Figure 8C. Network Protein Sequence analysis show that the μA protein can be arbitrarily divided into 4 regions. An amino terminal region spans residues 1 to 149, a variable region spans residues 150 to 462, a α-helix-rich region spans residues 463 to 615 and the carboxyl-terminal region spans residues 616 to 732. These designated regions are similar to those seen in the μ2 proteins of the three MRV serotypes. The

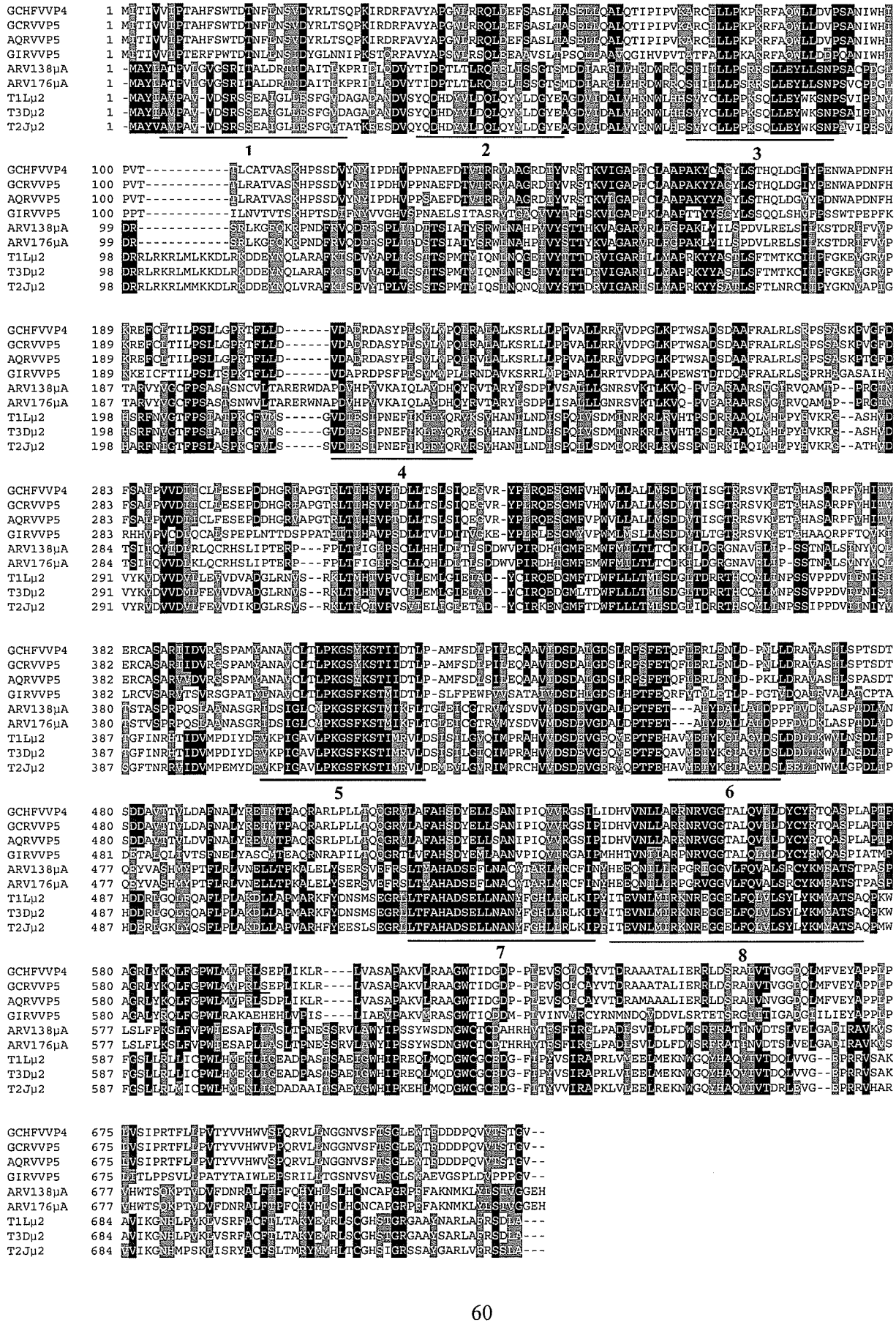

GCHFVVP4
GCRVVP5
AQRVVP5
GIRVVP5
ARV138μA
ARV176μA
T1Lμ2
T3Dμ2
T2Jμ2
1
2
3
4
5
6
7
8

Figure 6. Alignment of the deduced μA amino acid sequences of ARV138 and ARV176. Clones are: MRV -Type 1 Lang (T1Lμ2) (GenBank No. AF461682), Type 2 Jones (T2Jμ2) (GenBank No. AF124519), and Type 3 Dearing (T3Dμ2) (GenBank No. AF461683), ARV- ARV138μA and ARV176μA (clone accession numbers as in legend to Figure 5), GoldenIde reovirus VP5 (GIRVVP5) (GenBank No. AAM93416), Aquareovirus C VP5 (AQRVVP5) (GenBank No. NP938064), Grass Carp reovirus VP5 (GCRVVP5) (GenBank No. AAM92736), and Grass carp hemorrhagic fever virus VP4 (GCHFVVP4) (GenBank No. AAG17627). Amino acid residues that are identical in at least five of the sequences are indicated by black background shading. Grey background shading indicates conservative amino acid substitutions in at least five of the sequences. Conserved regions 1-8 as identified by Yin *et al.*, 2004 are represented by numbers; region number 5 is represented by a red line; conserved region 6 by a blue line. The single letter amino acid code is used.

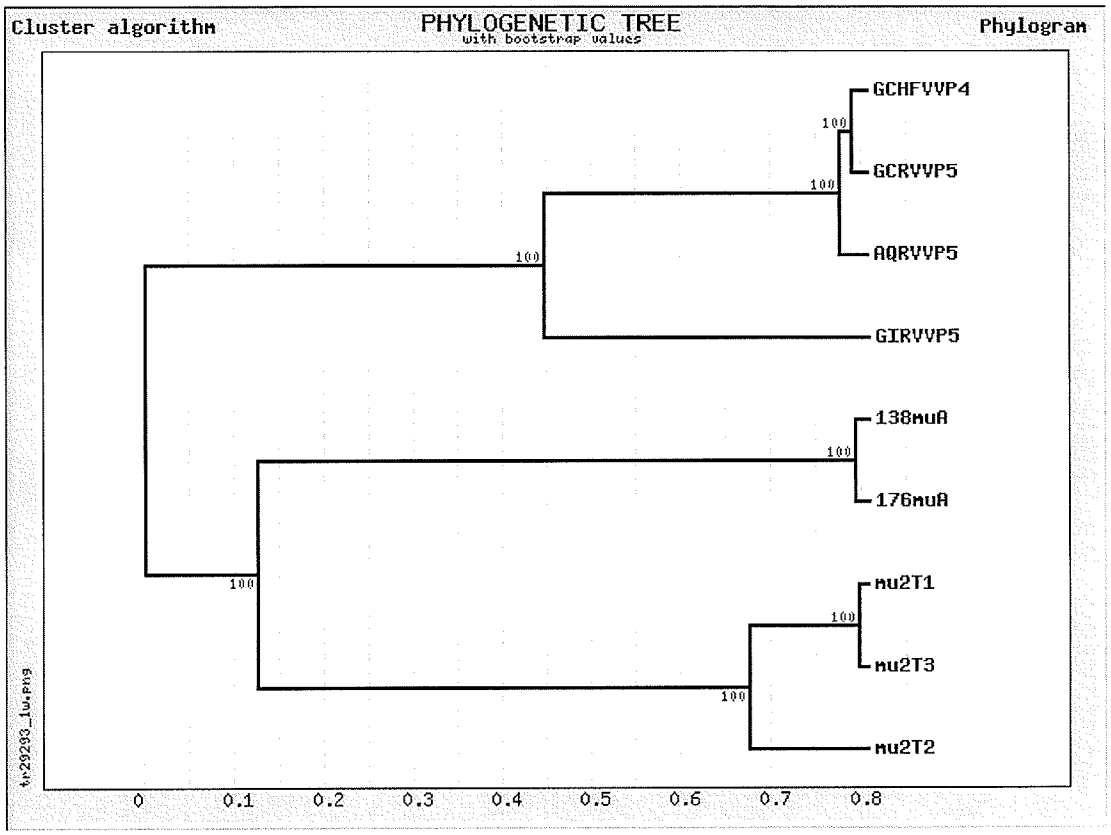
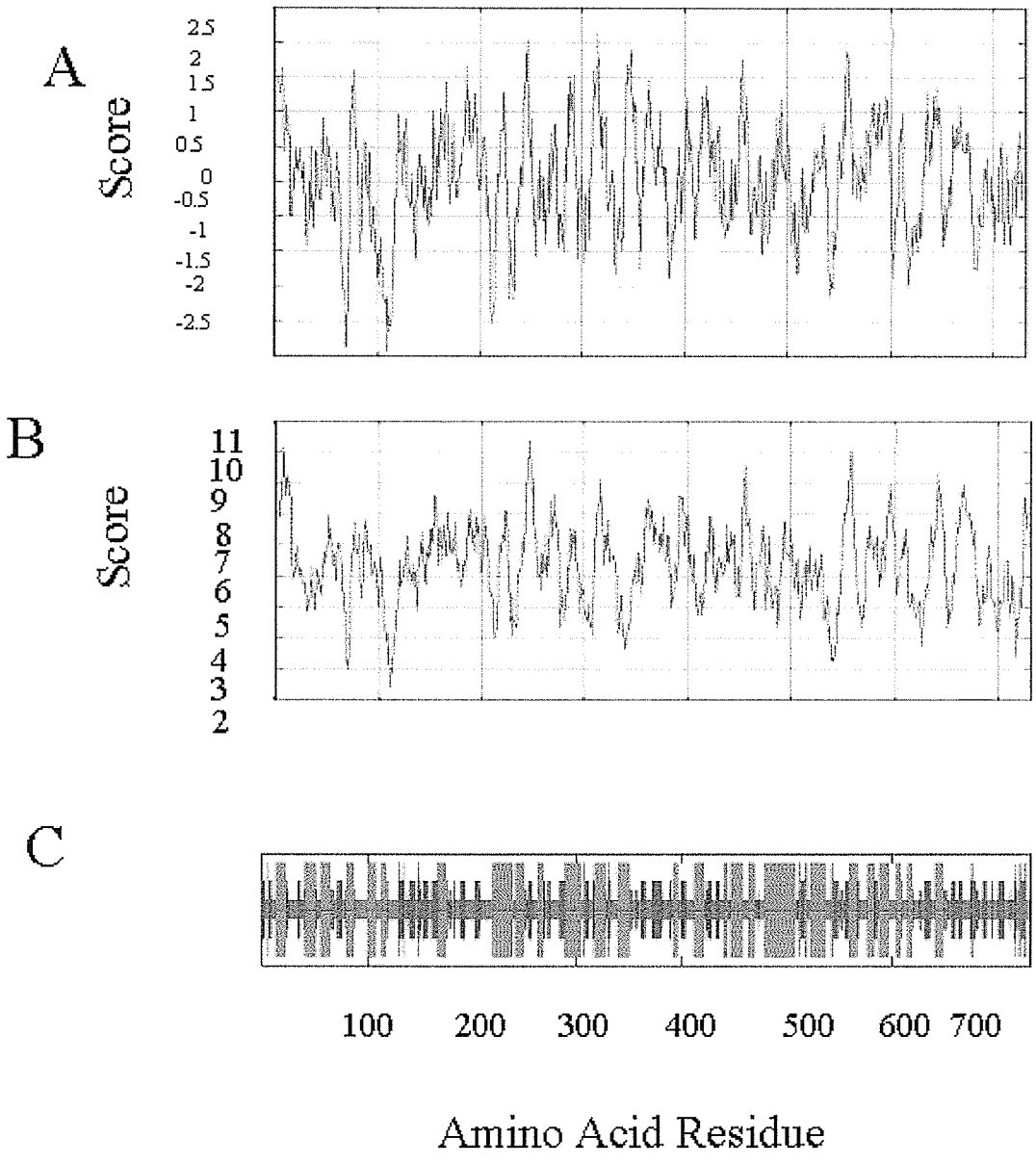

Cluster algorithm
PHYLOGENETIC TREE
with bootstrap values
Phylogram
GCHFVVP4
GCRVVP5
AQRVVP5
GIRVVP5
138muA
176muA
mu2T1
mu2T3
mu2T2
100
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Figure 7. Phylogenetic Tree Analyses of the prototype ARV deduced μA proteins and homologous proteins in other reoviruses, with homology as determined by BLAST analysis. Abbreviations are as follows: GCHFVVP4: Grass Carp Hemorrhagic Fever Virus VP4, GCRVVP5: Grass Carp Reovirus VP5, AQRVVP5: Aquareovirus C VP5, GIRVVP5: GoldenIde Reovirus VP5, mu2T1: MRV Type 1 Lang μ2, mu2T3: MRV Type 3 Dearing μ2, mu2T1: MRV Type 2 Jones μ2, 176muA: ARV 176 μA, 138muA: ARV 138 μA. GenBank numbers can be found in the legend to Figure 6. Horizontal lines are proportional in length to nucleotide substitution. Bootstrap numbers are presented in red, expressed in percentages and placed at the nodes of the tree. Analyses were performed using the default settings of the TreeTop- Phylogenetic Tree Prediction Software provided by GeneBee.

http://www.genebee.msu.su/services/phtree_reduced.html.


A
Score
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
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Score
11
10
9
8
7
6
5
4
3
2
C
100
200
300
400
500
600
700
Amino Acid Residue

Figure 8. Secondary structure predictions of μA proteins. Predictions of the ARV138 and ARV176 proteins were virtually identical; thus, only ARV138 predictions are shown. **A)** Hydropathicity index predictions, determined by the Kyte-Doolittle method (Kyte and Doolittle, 1982), using ExPasy Molecular Biology Server Protscale, a window length of 9 and a linear weight variation model.

B) Surface probability predictions, % buried residues determined using the ExPasy Molecular Biology Server, ProtScale.

C) Predicted secondary structures, alpha-helices represented by the tall blue lines, extended beta-sheets indicated by the medium red lines, and beta-turns represented by the shortest green lines. Predictions were made using the Network Protein Sequence Analysis Server, provided by Pole Bio-Informatique Lyonnais.

amino terminal region (a.a. 1-149) contains six α-helices and six β–sheets and is highly conserved amongst the two ARV serotypes. The variable region (a.a. 150-462) is the most structurally divergent region among the ARV serotypes and also contains several regions that are highly conserved amongst both ARV serotypes, as well as the three MRV serotypes. The third region (a.a. 463-615) is α-helix rich, containing seven α-helices and is also highly conserved. The carboxyl-terminal region (a.a. 616-732) is highly variable between the two ARV serotypes. Overall, the ARV μA protein is approximately 38% α-helix, 20% β–sheet and 35% random coil, making it an "alpha-beta" protein according to the CATH designation (Martin *et al.,* 1998). MOTIF and FingerPRINTScan programs were used to compare the ARV μA sequences with other sequences in protein data banks (ProSite, Blocks, and ProDomain). This comparison suggested that no currently recognized motifs (i.e. N-glycosylation sites, Zn^{2+} finger motif) or runs of sequence are present in either ARV μA protein. An NTPase domain was identified by Yin *et al.*, 2004 in MRV μ2 ; this motif is not present in the ARV μA sequence.

3.2.2 Comparisons of the μB protein sequences

As shown in Table 3, the encoded μB proteins are predicted to have molecular weights of 73.1 kDa (ARV138) and 73.3 kDa (ARV176). The predicted isoelectric points of the two ARV μB proteins are 5.24 (ARV138) and 5.53 (ARV176). The ARV μB proteins share approximately 95% homology (34 substitutions out of 676 amino acids). The majority of the amino acid substitutions result in a conservative change to an amino acid of similar size and charge (data not shown). Alignment of the MRV μ1 protein and the ARV μB proteins showed that the two proteins share approximately 45% amino acid identity (data not shown). Studies of the ARV M2 gene suggest that differences in the μB

sequence may be responsible for strain-specific efficiency of infection (O'Hara *et al.*, 2001). The MRV μ1 protein is the major outer capsid protein, playing a role in cellular penetration, and the regions of protein sequence similarity between the ARV and MRV proteins encoded by the M2 genes suggests the ARV μB protein may have similar activities. BLAST analysis suggests lesser amino acid sequence identity with several aquareovirus proteins and alignment of the aquareovirus, MRV and ARV proteins demonstrates several conserved regions (Figure 9). Alignment of the aquareovirus proteins with μB/μ1 demonstrates the almost complete conservation of the myristoylation site, as well as the μ1N/μ1C cleavage site. The conservation of these sites suggests that the myristoylation and cleavage of these proteins play key roles in the function of the protein, and they may all have similar roles in viral maturation and entry. The phylogenetic analysis shown in Figure 10 demonstrates a closer phylogenetic relationship between the ARV and MRV reoviruses, than the ARV and aquareoviruses.

Predictions of μB secondary structure (using the Kyte-Doolittle regional hydropathy, ExPasy ProtScale surface probability and Network Protein Sequence Analysis) were also determined. The ARV138 and ARV176 hydropathy profiles, as well as surface probability profiles were identical and show numerous regions that are predicted to be exposed at the surface of the protein as well as several regions predicted to be buried (Figure 11A and B). The predicted secondary structure of the ARV μB protein is shown in Figure 11C. Network Protein Sequence analysis show that the μB protein can be arbitrarily divided into 4 regions. An amino terminal region spans residues 1 to 105, a α-helix-rich region spans residues 106 to 300, a variable region spans residues 301 to 413 and the carboxyl-terminal region, which is α-helix-rich, spans residues 414 to

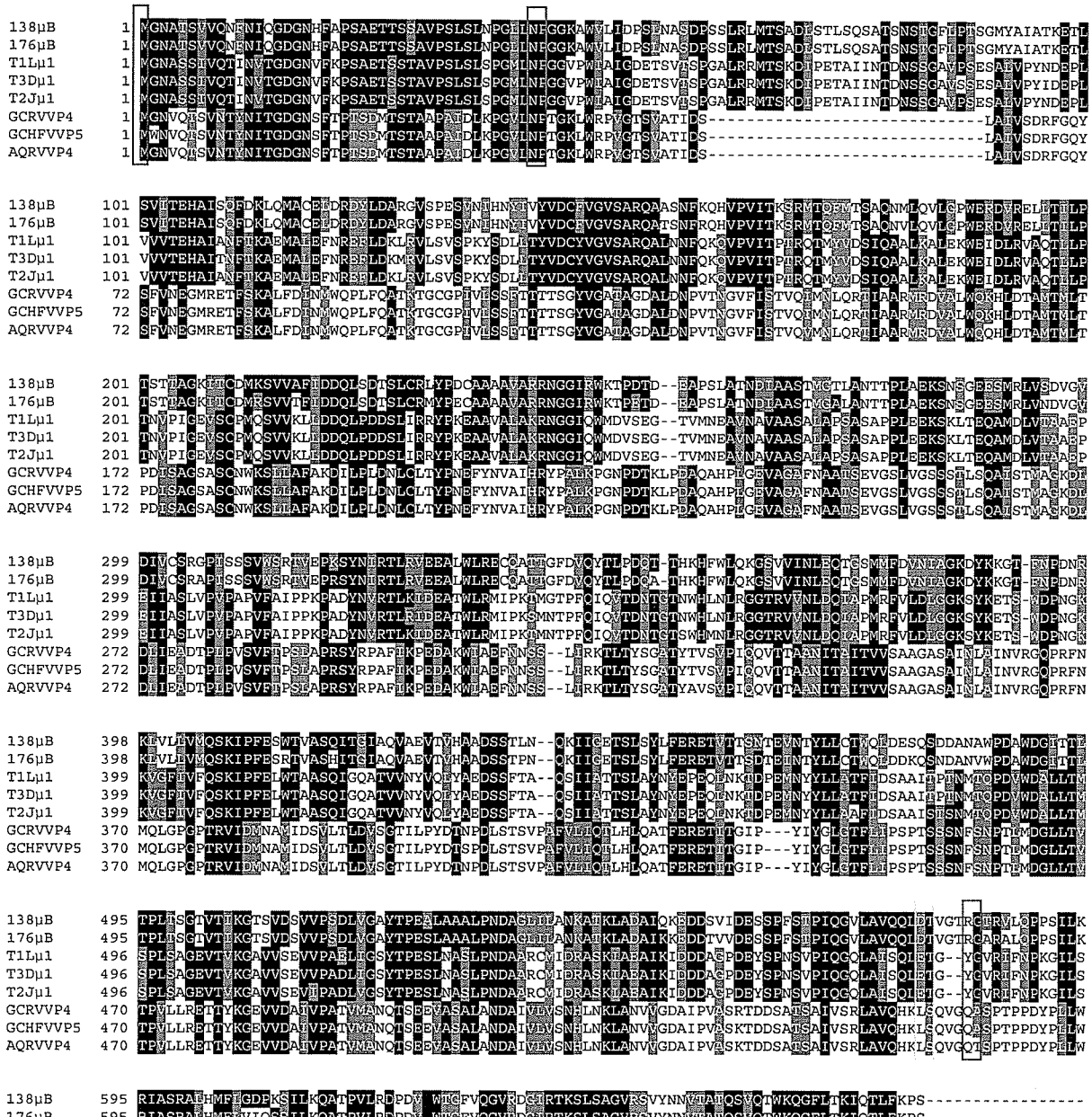
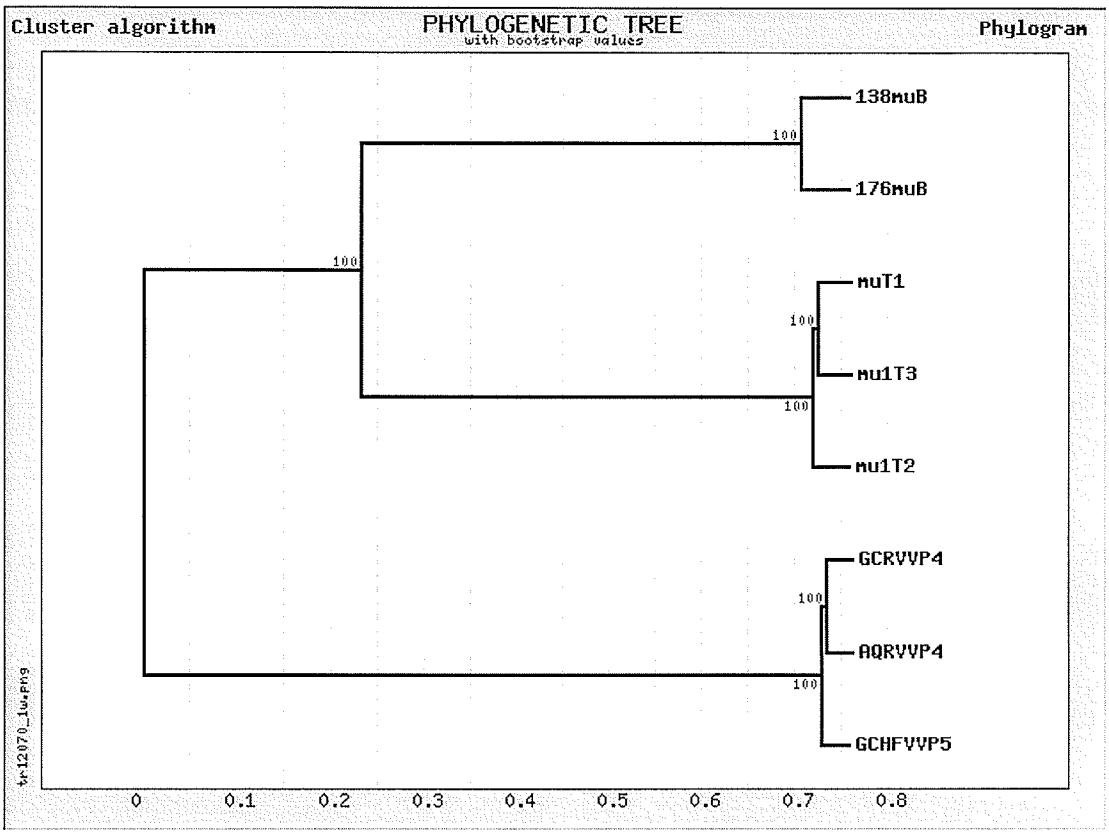


Figure 9. Alignment of the deduced μB amino acid sequences of ARV138 and ARV176. Clones are: MRV -Type 1 Lang (T1Lμ1) (GenBank No. AAM10735), Type 2 Jones (T2Jμ1) (GenBank No. NC_004270), and Type 3 Dearing (T3Dμ1) (GenBank No. NC_004278), ARV- ARV138μB (GenBank AY750052) and ARV176μB (GenBank No. AY750053), Aquareovirus C VP4 (AQRVVP4) (GenBank No. AAM92749), Grass Carp reovirus VP4 (GCRVVP4) (GenBank No. NP_938001), and Grass carp hemorrhagic fever virus VP5 (GCHFVVP5) (GenBank No. AF239175). The shading scheme is the same as in Figure 6. The N-terminus myristoylation is represented by a blue box, the μ1N/μ1C cleavage site is represented by a red box, the δ/ϕ site by chymotrypsin is represented by a yellow box and the δ/ϕ site by trypsin is represented by a turquoise box. The single letter amino acid code is used.


Cluster algorithm
PHYLOGENETIC TREE
with bootstrap values
Phylogram
138muB
176muB
muT1
mu1T3
mu1T2
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AQRVVP4
GCHFVVP5
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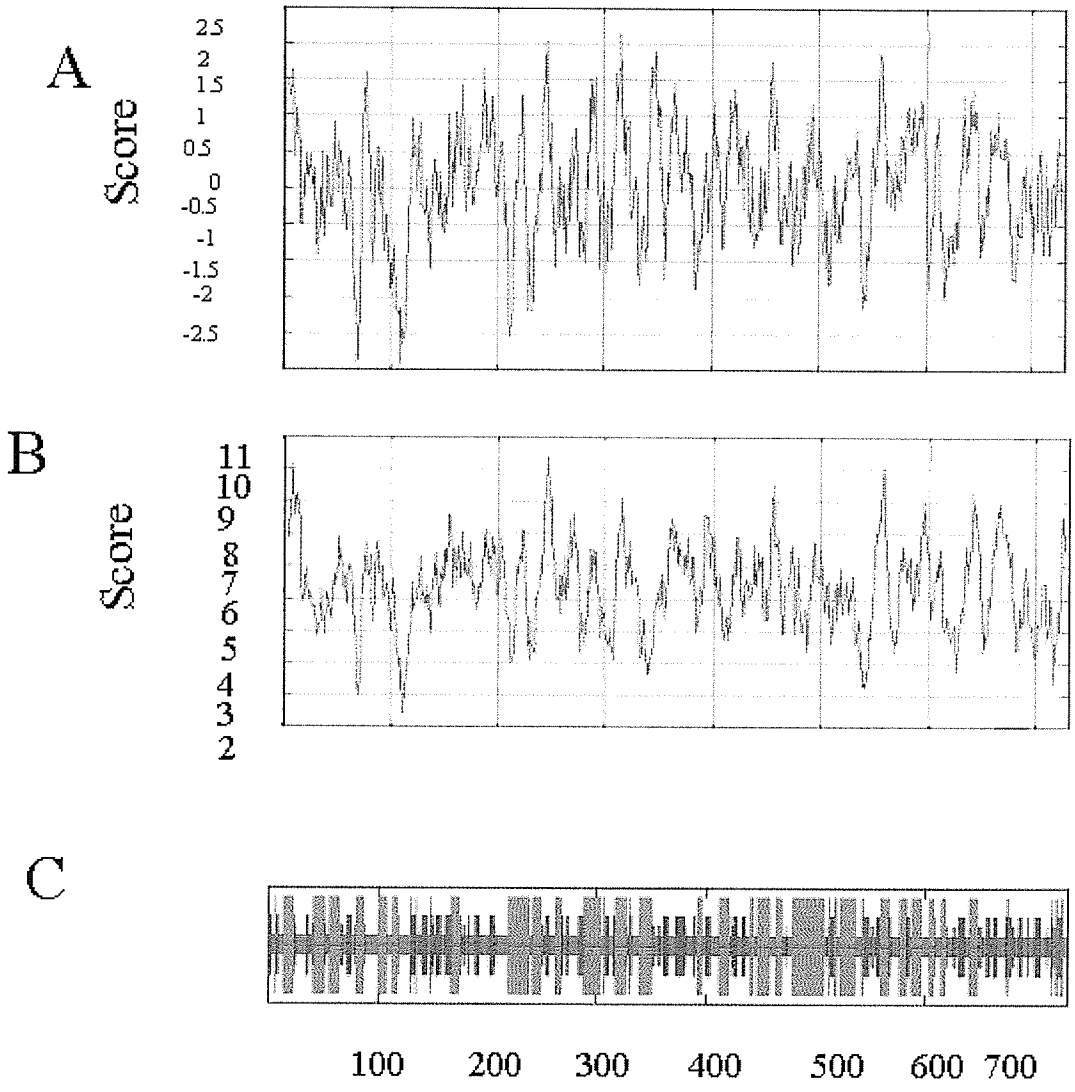
100
100
100
100
100
tr12070_1w.png
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Figure 10. Phylogenetic Tree Analyses of the prototype ARV deduced μB proteins and homologous proteins in other reoviruses, with homology as determined by BLAST analysis. Abbreviations are as follows: 138mub: ARV138 μB, 176mub: ARV176 μB, mu1T1: MRV Type 1 Lang μB, mu1T3: MRV Type 3 Dearing μB, mu1T2: MRV Type 2 Jones μB, AQRVVP4: Aquareovirus C VP4, GCRVVP4: Grass Carp Reovirus VP4, GCHFVP5: Grass Carp Hemorrhagic Fever Virus VP5. GenBank numbers can be found in the legend to Figure 9. Horizontal lines are proportional in length to nucleotide substitution. Bootstrap numbers are presented in red, expressed in percentages and placed at the nodes of the tree. Analyses were performed using the default settings of the TreeTop- Phylogenetic Tree Prediction Software provided by GeneBee. http://www.genebee.msu.su/services/phtree_reduced.html.


A
Score
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
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Score
11
10
9
8
7
6
5
4
3
2
C
100
200
300
400
500
600
700
Amino Acid Residue

Figure 11. Secondary structure predictions of μB proteins. Predictions of the ARV138 and ARV176 proteins were virtually identical; thus, only ARV138 predictions are shown.

A) Hydropathicity index predictions, determined by the Kyte-Doolittle method (Kyte and Doolittle, 1982), using ExPasy Molecular Biology Server Protscale, a window length of 9 and a linear weight variation model.

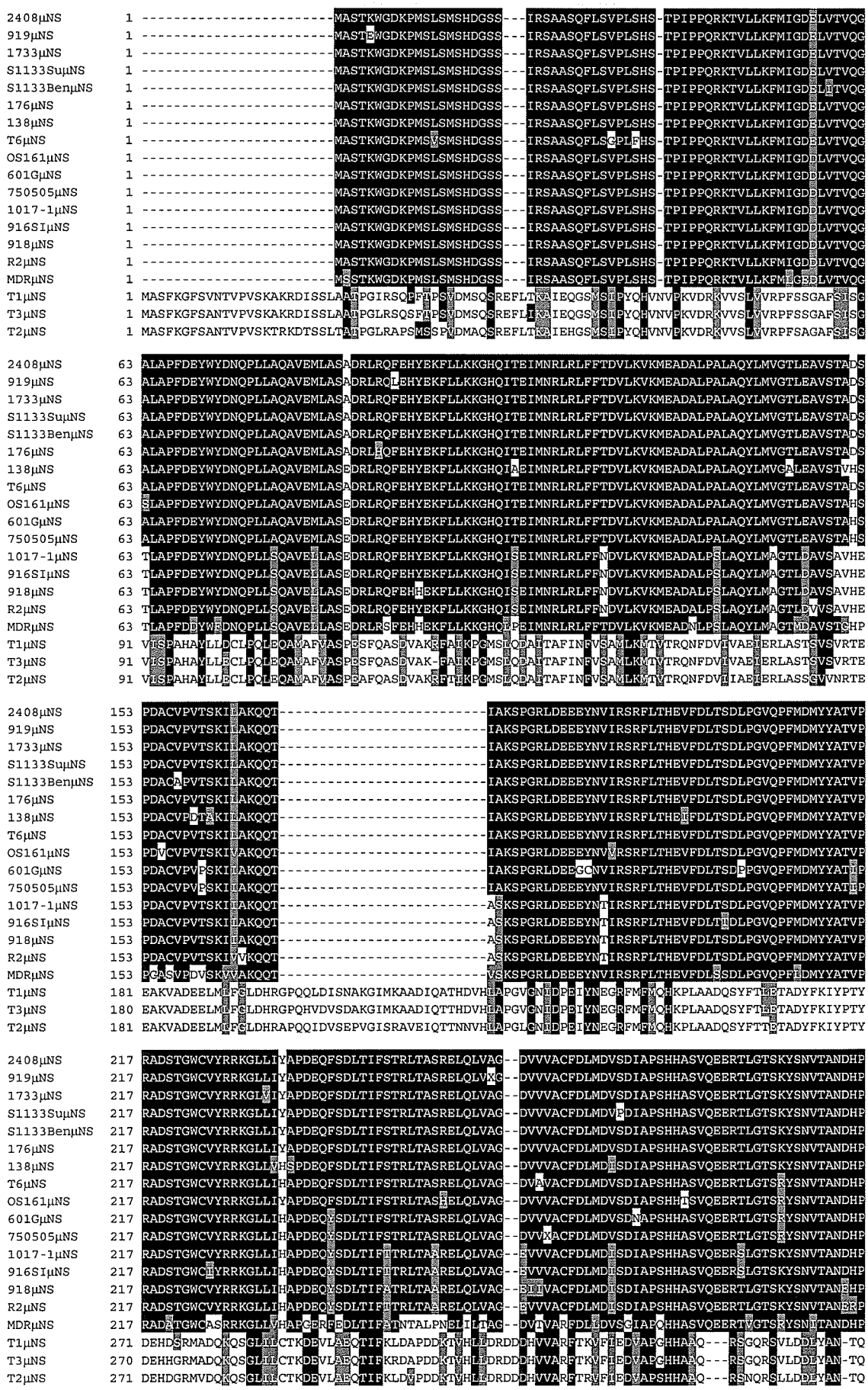
B) Surface probability predictions, % buried residues determined using the ExPasy Molecular Biology Server, ProtScale.

C) Predicted secondary structures, alpha-helices represented by the tall blue lines, extended beta-sheets indicated by the medium red lines, and beta-turns represented by the shortest green lines. Predictions were made using the Network Protein Sequence Analysis Server, provided by Pole Bio-Informatique Lyonnais.

676. Overall, the ARV μB protein is approximately 38% α-helix, 20% β–sheet and 35% random coil, making it an "alpha-beta" protein according to the CATH designation (Martin *et al.,* 1998). MOTIF and FingerPRINTScan programs were used to compare the ARV μB sequences with other sequences in protein data banks (ProSite, Blocks, and ProDomain). This comparison suggested that no currently recognized motifs or runs of sequence are present in either ARV μB protein.

3.2.3 Comparisons of the μNS protein sequences

The ARV μNS proteins are composed of 635 amino acids, making them 84 amino acids shorter than the homologous MRV protein. The molecular weight of the ARV138 μNS protein is 70.9 kDa and the molecular weight of the ARV176 μNS protein is 70.8 kDa (Table 3). The two ARV proteins sequenced here have approximately 97% identity, with 20 amino acid differences between them. The majority of these amino acid substitutions result in conservative changes to amino acids of similar charges and size (data not shown). The predicted isoelectric points of the ARV μNS proteins are 5.92 (ARV138) and 5.88 (ARV176). Alignment of ARV138 and ARV176 μNS proteins with the recently determined ARV 1733 μNS protein showed approximately 99% homology between ARV176 μNS and ARV 1733 μNS and approximately 96% between ARV138 μNS and ARV 1733 μNS. Alignment of the MRV μNS protein and the ARV μNS proteins showed that the two sets of proteins share approximately 25% amino acid identity (Figure 12). This low level of conservation between MRV and ARV, as well as the large size difference between the proteins suggest that the μNS proteins may have different roles in ARV and MRV. BLAST searches demonstrated sequence homology between the two ARV strains' μNS proteins sequenced in this study, the μNS proteins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919μNS
1733μNS
S1133SuμNS
S1133BenμNS
176μNS
138μNS
T6μNS
OS161μNS
601GμNS
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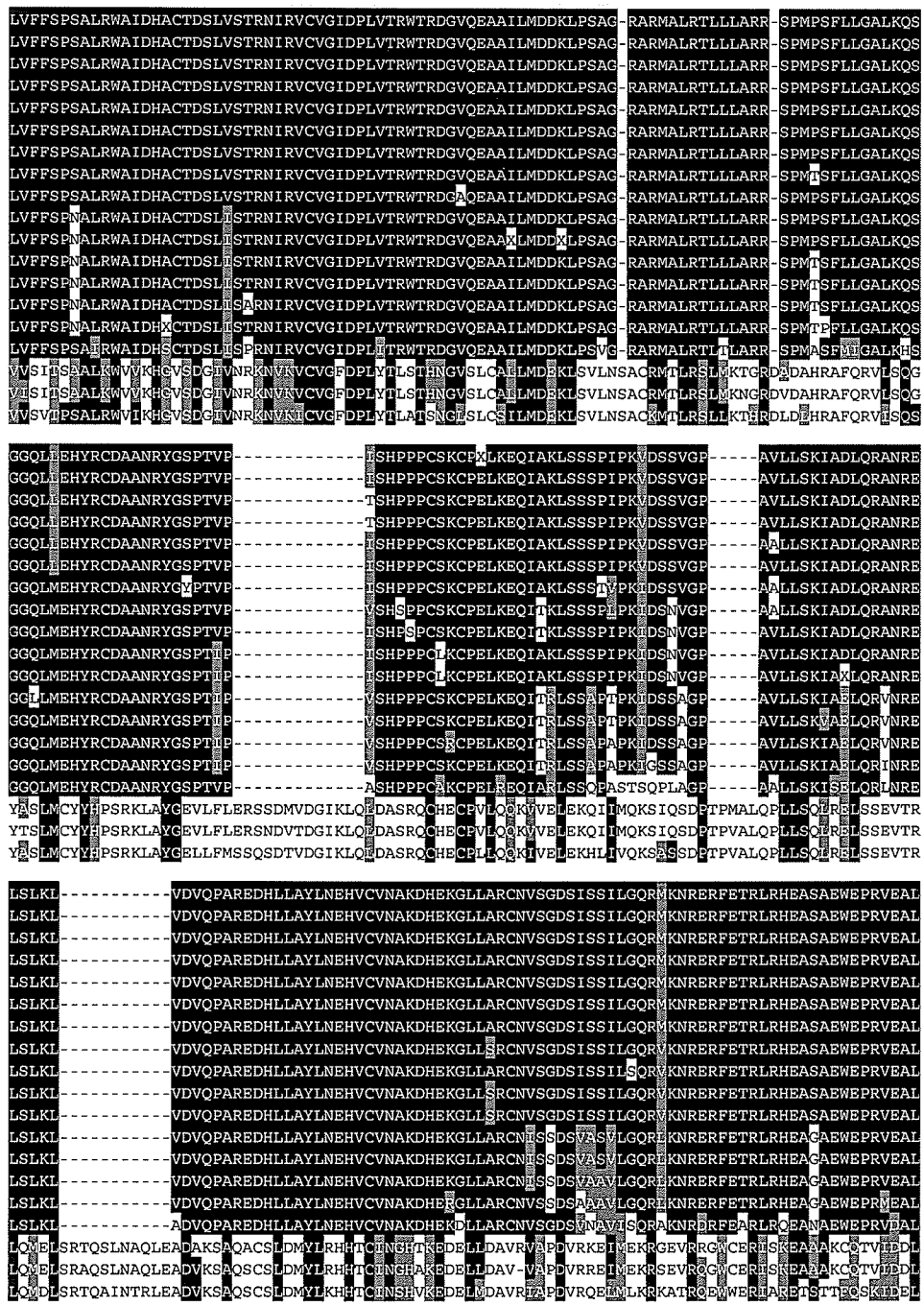
1017-1μNS
916SIμNS
918μNS
R2μNS
MDRμNS
T1μNS
T3μNS
T2μNS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919μNS 305
1733μNS 305
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601GμNS 305
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916SIμNS 305
918μNS 305
R2μNS 305
MDRμNS 305
T1μNS 357
T3μNS 356
T2μNS 357
2408μNS 393
919μNS 393
1733μNS 393
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S1133BenμNS 393
176μNS 393
138μNS 393
T6μNS 393
OS161μNS 393
601GμNS 393
750505μNS 393
1017-1μNS 393
916SIμNS 393
918μNS 393
R2μNS 393
MDRμNS 393
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T6μNS 465
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601GμNS 465
750505μNS 465
1017-1μNS 465
916SIμNS 465
918μNS 465
R2μNS 465
MDRμNS 465
T1μNS 537
T3μNS 536
T2μNS 537
2408μNS 544
919μNS 544
1733μNS 544
S1133SuμNS 544
S1133BenμNS 544
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T3μNS 625
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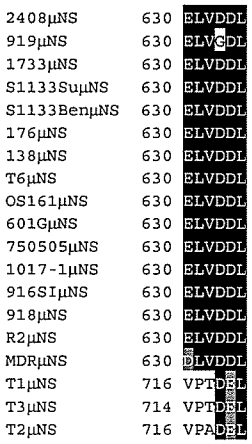
2408μNS 630 ELVDDL
919μNS 630 ELVGDL
1733μNS 630 ELVDDL
S1133SuμNS 630 ELVDDL
S1133BenμNS 630 ELVDDL
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138μNS 630 ELVDDL
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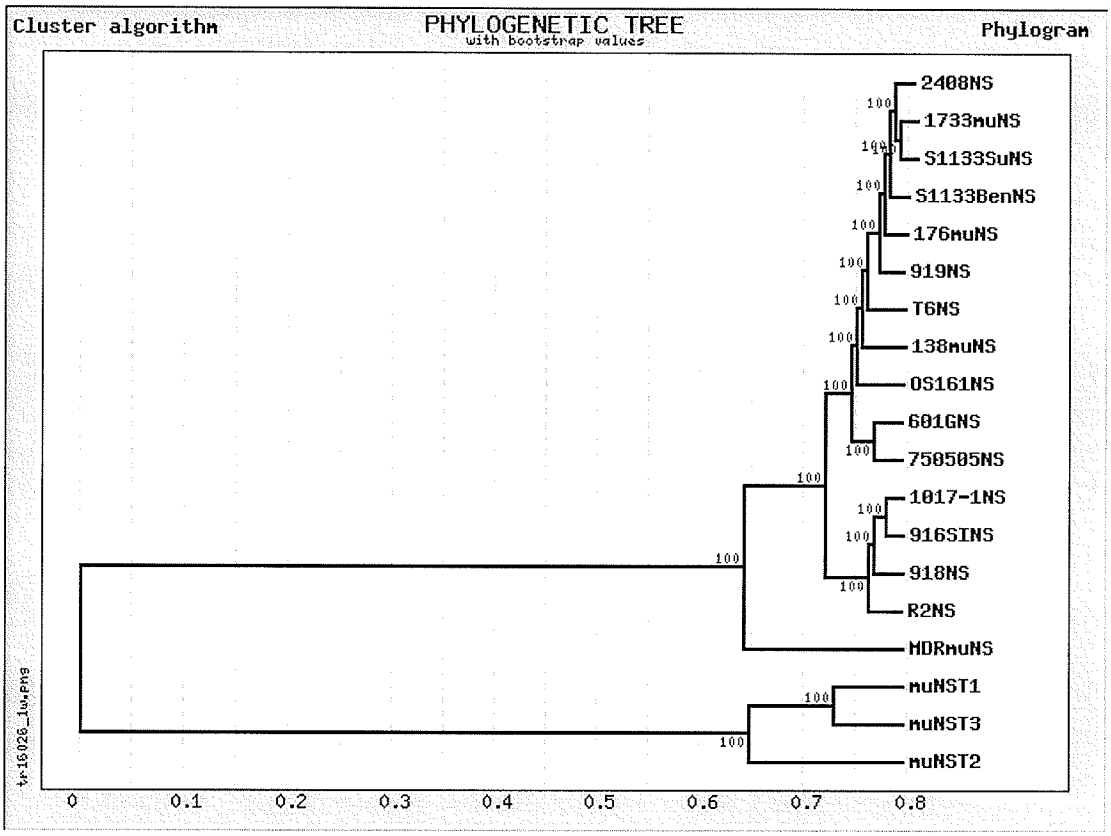
750505μNS 630 ELVDDL
1017-1μNS 630 ELVDDL
916SIμNS 630 ELVDDL
918μNS 630 ELVDDL
R2μNS 630 ELVDDL
MDRμNS 630 DLVDDL
T1μNS 716 VPTDEL
T3μNS 714 VPTDEL
T2μNS 716 VPADEL

Figure 12. Alignment of the deduced μNS amino acid sequences of ARV138 and ARV176. Abbreviations are as follows: ARV- ARV138μNS (138μNS) (GenBank No. AY557190), ARV176 μNS (176μNS) (GenBank No. AY557191), ARV1733 (1733NS) (GenBank No. AY303993), "extended" Muscovy Duck Reovirus (MDRμNS) (see text for description of sequence determination) (GenBank No. AJ293969), Avian S1133 2408 μNS (2408μNS) (GenBank No. AAS78990), Avian S1133 919 μNS (919μNS) (GenBank No. AAS78995), Avian S1133 μNS (S1133SuμNS) (Su *et al.*, 2004) (GenBank No. AAS78987), Avian S1133 μNS (Touris-Otero *et al.*, 2004) (S1133BenμNS) (GenBank No. AAT85608), Avian S1133 T6 μNS (T6μNS) (GenBank No. AAS78998), Avian S1133 OS161 μNS (OS161μNS) (GenBank No. AAS78996), Avian S1133 601G μNS (601GμNS) (GenBank No. AAS78991), Avian S1133 750505 μNS (750505GμNS) (GenBank No. AAS78992), Avian S1133 1017-1 μNS (1017-1μNS) (GenBank No. AAS78988), Avian S1133 916SI μNS (916SIμNS)- (GenBank No. AAS78993), Avian S1133 918 μNS (918μNS) (GenBank No. AAS78994), Avian S1133 R2 μNS (R2μNS) (GenBank No. AAS78997), MRV -Type 1 Lang (T1μNS) (GenBank No. NP694610), Type 2 Jones (T2μNS) (GenBank No. NP694611), and Type 3 Dearing (T3μNS) (GenBank No. NP694686). The shading scheme is the same as in Figure 6. The single letter amino acid code is used.

from thirteen sequenced ARV S1133 strains, three MRV strains' μNS proteins and Muscovy Duck Reovirus μNS protein (MDR). It has been proposed that the published sequence of MDR M3 (GenBank No. AJ293969) contains an insertion between positions ^{1666}ACACAATCCCC^{1675} which creates a μNS protein 83 residues shorter than the ARV μNS proteins (Touris-Otero, 2004, Dr. Roy Duncan, personal communication). Removal of one nucleotide from region 1666-1675 creates a 635 amino acid protein which corresponds well with the ARV μNS proteins. In order to perform further analysis with the MDR μNS this "extended" version of the protein was utilized. Phylogenetic analysis demonstrates the relationship between the predicted μNS proteins of the MRV, MDR and ARV (Figure 13). The μNS protein in MRV plays a role in transcription and may also play a role in replication. BLAST searches revealed similarity between the MRV and ARV proteins but no homology was found between the ARV μNS proteins and any other protein sequences in the database. Aquareoviruses have similar virus structures which appear to have been conserved from ARV and MRV. A number of different non-structural proteins have been identified for the aquareoviruses and the lack of sequence conservation in the non-structural proteins suggests there may be differences in the replication cycle of the aquareoviruses and ARV/MRV. Predictions of μNS secondary structure were determined using ExPasy ProtScale surface predictions, Kyte & Doolittle regional hydropathy scale and Network Protein Sequence Analysis Programs. The ARV 138 and ARV 176 μNS hydropathy profiles and surface probability profiles were identical and show numerous regions that are predicted to be exposed at the surface of the protein as well as several regions predicted to be buried (Figure 14A and B). The predicted secondary structure of the ARV μNS protein can be seen in Figure 14C.


Cluster algorithm
PHYLOGENETIC TREE
with bootstrap values
Phylogram
2408NS
1733muNS
S1133SuNS
S1133BenNS
176muNS
919NS
T6NS
138muNS
OS161NS
601GNS
758585NS
1017-1NS
916SINS
918NS
R2NS
MDRmuNS
muNST1
muNST3
muNST2
0
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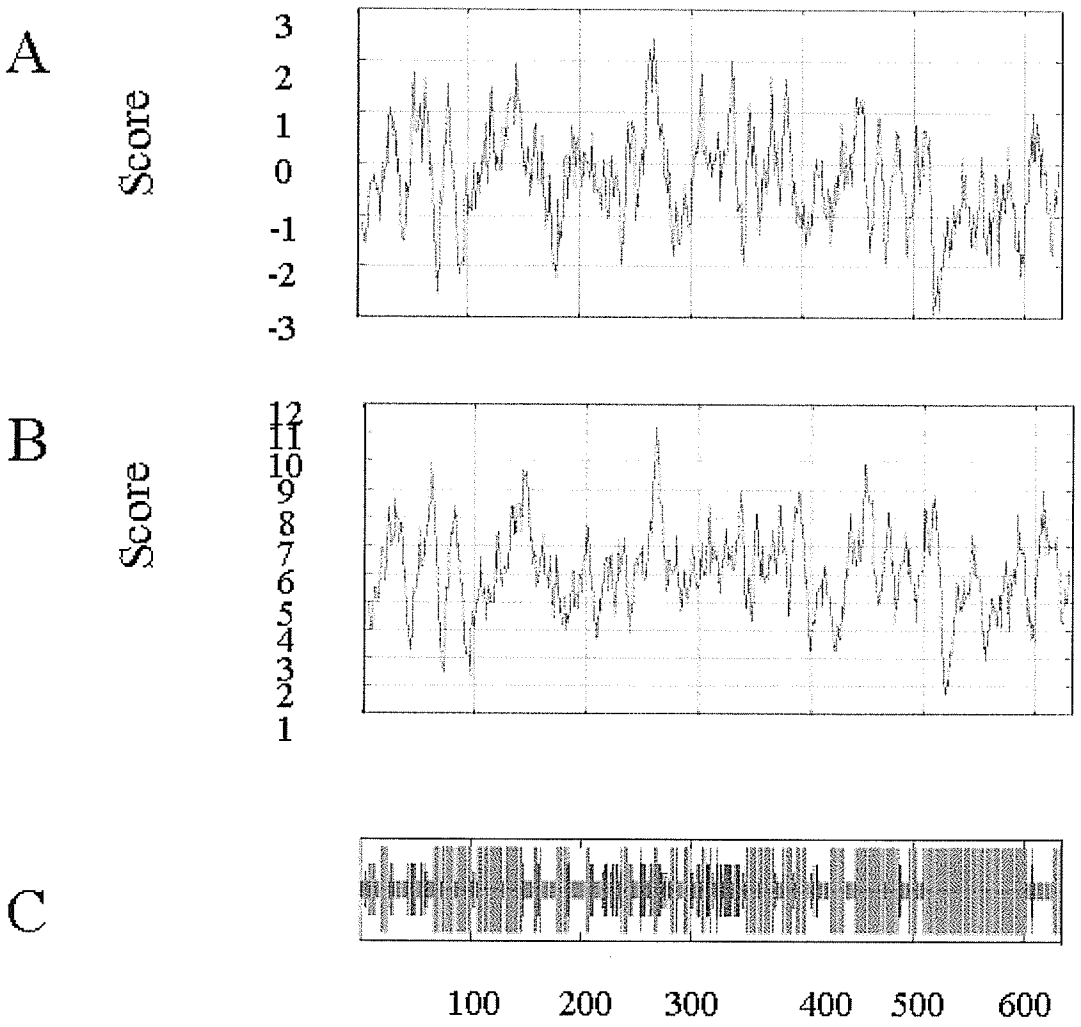
0.2
0.3
0.4
0.5
0.6
0.7
0.8
tr16026_lw.phs

Figure 13. Phylogenetic Tree Analyses of the prototype ARV deduced μNS proteins and homologous proteins in other reoviruses, with homology as determined by BLAST analysis. Abbreviations are as follows: 138NS: ARV138 μNS, 176NS: ARV176 μNS, 1733NS: ARV1733 μNS, 2048NS: Avian S1133 2408 μNS, 919NS: Avian S1133 919 μNS,1733 NS: Avian S1133 1733 μNS, S1133SuNS: Avian S1133 μNS, S1133BenNS: Avian S1133 μNS, T6NS: Avian S1133 T6 μNS, OS161NS: Avian S1133 OS161 μNS, 601GNS: Avian S1133 601G μNS, 750505GNS: Avian S1133 750505 μNS, 1017-1NS: Avian S1133 1017-1 μNS, 916SINS: Avian S1133 916SI μNS, 918NS: Avian S1133 918 μNS, R2NS: Avian S1133 R2 μNS, MRDNS: MDR μNS, T1NS: MRV Type 1 Lang μNS, T3NS: MRV Type 3 Dearing μNS, T2NS: MRV Type 2 Jones μNS. GenBank numbers can be found in the legend to Figure 12. Horizontal lines are proportional in length to nucleotide substitution. Bootstrap numbers are presented in red, expressed in percentages and placed at the nodes of the tree. Analyses were performed using the default settings of the TreeTop- Phylogenetic Tree Prediction Software provided by GeneBee. http://www.genebee.msu.su/services/phtree_reduced.html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Score
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0
-1
-2
-3
B
Score
12
11
10
9
8
7
6
5
4
3
2
1
C
100
200
300
400
500
600
Amino Acid Residue

Figure 14. Secondary structure predictions of μNS proteins. Predictions of the ARV138 and ARV176 proteins were virtually identical; thus, only ARV138 predictions are shown.

A) Hydropathicity index predictions, determined by the Kyte-Doolittle method (Kyte and Doolittle, 1982), using ExPasy Molecular Biology Server Protscale, a window length of 9 and a linear weight variation model.

B) Surface probability predictions, % buried residues determined using the ExPasy Molecular Biology Server, ProtScale.

C) Predicted secondary structures, alpha-helices represented by the tall blue lines, extended beta-sheets indicated by the medium red lines, and beta-turns represented by the shortest green lines. Predictions were made using the Network Protein Sequence Analysis Server, provided by Pole Bio-Informatique Lyonnais.

Network Protein Sequence analysis show that the μNS protein can be arbitrarily divided into 5 regions. An amino terminal region spans residues 1 to 68, a α-helix-rich region spans residues 69-209, a variable region spans residues 210 to 349, a second α-helix-rich region spans residues 350 to 603 and the carboxyl-terminal region spans residues 604 to 635. The amino terminal region (a.a. 1-68) contains one α-helix and four β–sheets and is highly conserved amongst the two ARV serotypes. The first α-helix rich region (a.a. 69-209) contains ten α-helices, which are largely conserved, and three β–sheets. The variable region (a.a. 210-349) is the most structurally divergent region among the ARV serotypes and also contains several regions that are highly conserved amongst both ARV serotypes, as well as the three MRV serotypes. The second α-helix rich region (a.a. 350-603) contains ten α-helices, of varying length, and is also highly conserved. The carboxyl-terminal region (a.a. 604-635) is highly variable between the two ARV serotypes. Overall, the ARV μNS protein is approximately 52% α-helix, 13% β–sheet and 30% random coil, making it a "mixed" protein according to the CATH designation (Martin *et al.,* 1998). The high helical content predicted in the μNS protein is similar to what is predicted for its MRV counterpart, the μNS protein (McCutcheon, 1999).

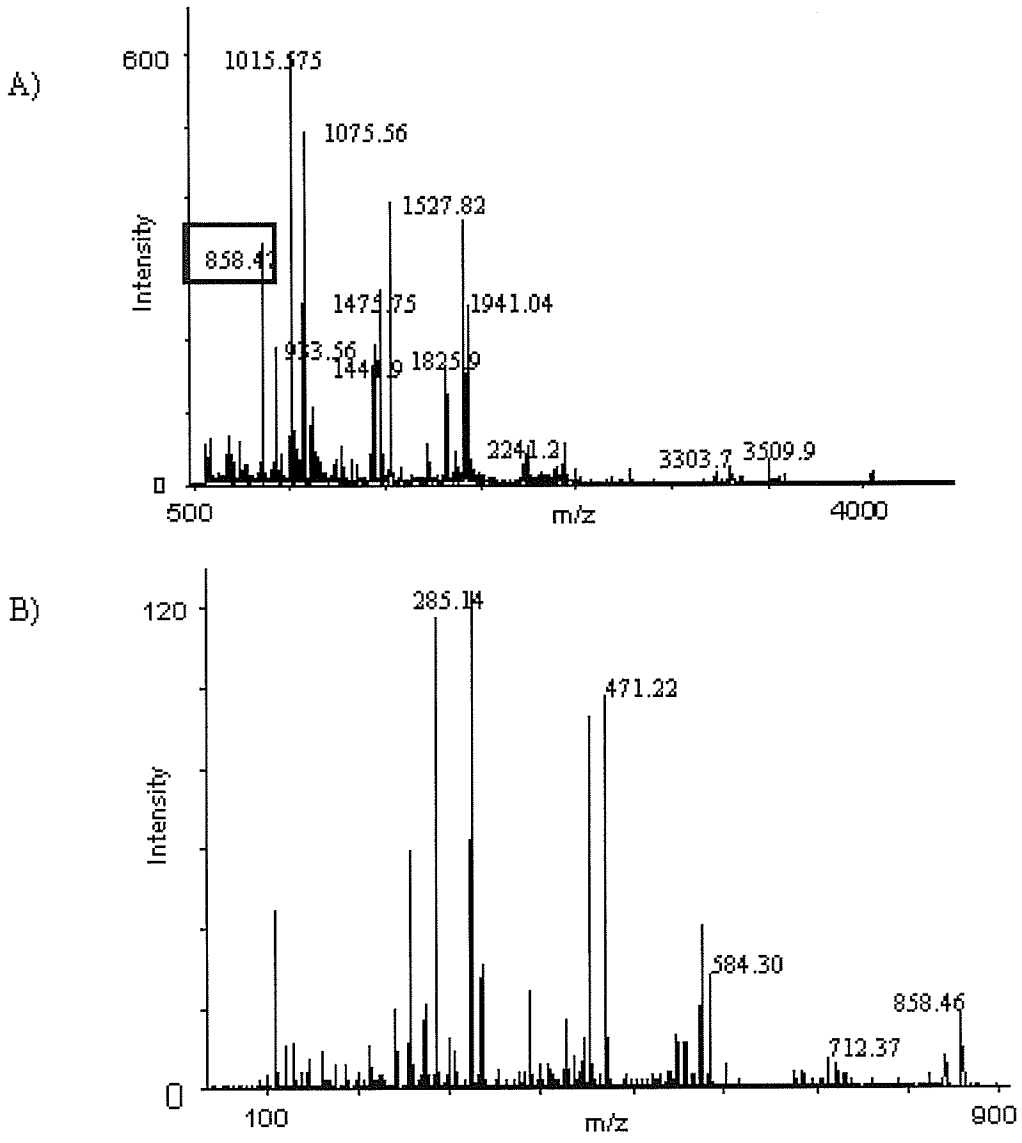
MOTIF and FingerPRINTScan programs were used to compare the ARV μNS sequences with other sequences in protein data banks (ProSite, Blocks, and ProDomain). This comparison suggested that no currently recognized motifs or runs of sequence are present in either ARV μNS protein. However, the tertiary structure of the protein could be predicted using SwissModel (data not shown), unlike the ARV μA and μB proteins, suggesting that there are runs of sequence which could potentially generate motifs. Either

the sequence runs or motifs appear to be recognized as putative tertiary structure by Swiss Model algorithms.

3.2.4 Mass spectrometric analysis of the ARV μA and μB proteins

Although nucleotide sequencing from RNA has become more accurate, there are some reports of errors in reovirus sequencing that resulted in frameshifts (Harrison *et al.*, 1999). Thus, I sought biochemical corroboration of our newly obtained predicted μA and μB protein sequences. The protein sequence of μNS could not be corroborated in this way, as it is a non-structural protein and can not be gel-purified. Since μB is a major outer capsid protein in virions, ARV138 virus particles were purified, the structural proteins were resolved by SDS-PAGE, and the μB protein band was subjected to in-gel tryptic digestion and mass spectrometry. Figure 15A is a representative spectrum showing 9 tryptic peptide ions ranging in measured sizes from 858 – 2440Da. Database searches indicated these peaks correspond to several different regions scattered throughout the μB sequence (from amino acids 65-675). Analyses of several spectra provided 54.6% coverage of the μB sequence. To confirm the database-predicted peptides, several of them were subjected to tandem mass spectrometry to directly sequence them (Figure 15B). Importantly, the identification of peptides throughout the μB sequence strongly suggests no frameshift errors in nucleotide determination and provides supporting evidence our sequences are correct.

Since μA is a minor core protein, it proved to be difficult to separate the protein from the μ proteins present in higher copy number. In order to separate the μA, purified ARV 138 was digested with α-chymotrypsin to obtain cores. The 5 core structural proteins were then resolved by SDS-PAGE, the μA band was excised and subjected to



HFWLQK(amino acids 357-362)

Figure 15. Mass spectrometric analyses of gel-purified ARV138 μB protein. A) MALDI-QqTOF mass spectrum obtained after digestion of gel-purified ARV138 μB with trypsin. Analysis of the peaks indicate peptides scattered throughout the region of amino acids 65-675 of the μB protein, suggesting the predicted sequence is correct and the M2 gene sequence does not contain any insertions or deletions. The peptides represent 54.6% coverage of the predicted μB protein sequence. Only a few representative peptides are labeled and shown here. **B)** The ion peak at m/z value 858.47 (red box) was dissociated using argon as the collision gas. Spectra data acquisition was performed using software developed in-house (Tofma, University of Manitoba, MB). Sequence analysis indicates the ion corresponds to μB peptide 357-362.

in-gel digestion and mass spectrometry. Analysis of the spectra (Figure 16) identified several peaks which provided approximately 23% coverage of the μA sequence (peptides scattered throughout amino acids 83-637). Identification of peptides throughout the μA sequence strongly suggests no frameshift errors in nucleotide determination and provides supporting evidence our sequences are correct.

3.3 Characterization of α-Reovirus Core Monoclonal Antibodies

3.3.1 α-Reovirus Core Monoclonal Antibodies

In an effort to gain further insight into the structure of the five reovirus core proteins, their individual functions and the activity of the entire core structure, monoclonal antibodies were produced to the core proteins. Monoclonal antibodies provide a unique binding specificity that can only bind a specific antigen. Minor amounts of cross-reactivity with other antigens containing similar epitopes may also be observed. The monoclonal antibodies produced here can be used in a wide variety of different ways to study the core proteins.

Traditionally, purified or recombinant protein is required to raise and characterize monoclonal antibodies and the process of obtaining the quantities of purified protein required to induce an immune response can be difficult. To that end, we have attempted to optimize an immunoprecipitation technique linked with mass spectrometry developed by Weiler *et al.* (2003) which eliminates the need for purified protein. In this method, antibodies are raised against a mixture of antigens and their specificity is characterized by mass spectrometric methods. In this study, dissociated core proteins were used to elicit the antibody response and immunoprecipitation, mass spectrometry, as well as more traditional immunological methods, such as ELISA and Western Blotting were used to

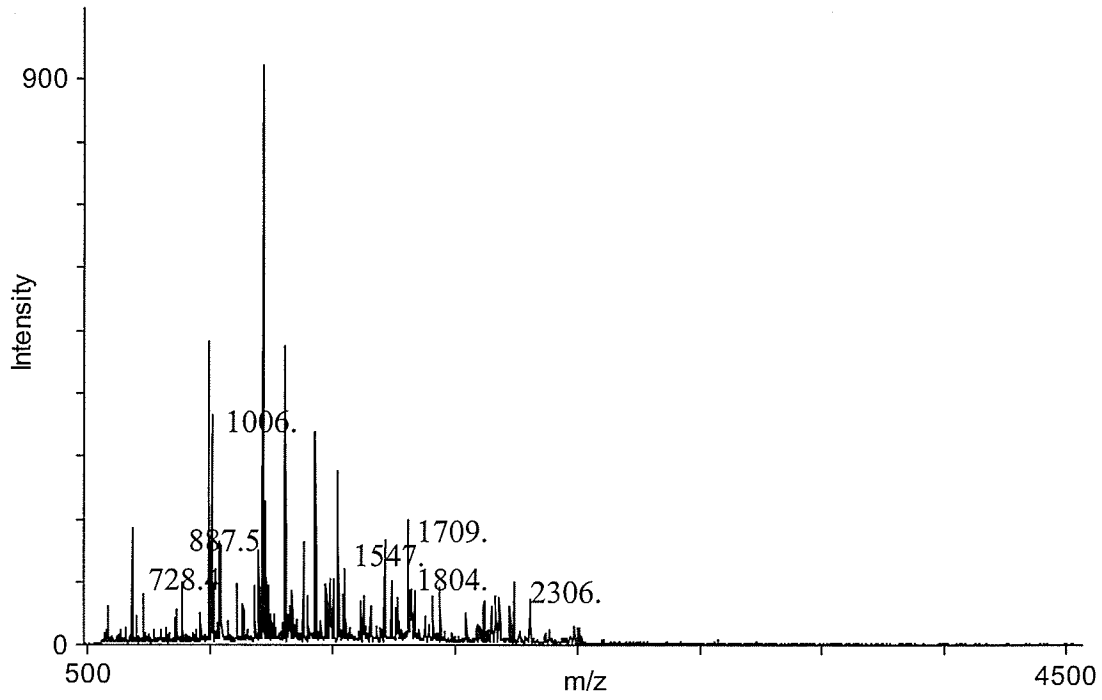

900
Intensity
0
500
m/z
4500
1006.
887.5
728.4
1547.
1709.
1804.
2306.

Figure 16. Mass spectrometric analyses of gel-purified ARV138 μA protein. The MALDI-QqTOF mass spectrum obtained after digestion of gel-purified ARV138 μA with trypsin. Spectra data acquisition was performed using software developed in-house (Tofma, University of Manitoba, MB). Analysis of the peaks indicate peptides scattered throughout the region of amino acids 83-637of the μA protein suggesting the predicted sequence is correct and the M2 gene sequence does not contain any insertions or deletions. The peptides represent 23% coverage of the predicted μA protein sequence.

characterize the specificity of two of the monoclonal antibodies raised.

3.3.2 Production of α-Reovirus Core Monoclonal Antibodies

In order to produce α-reovirus core protein antibodies, female BALB/c mice were subcutaneously immunized with dissociated reovirus cores mixed with a synthetic adjuvant, Titermax Gold. Four days before the harvesting of the spleen, each mouse was immunized intraperitoneally with dissociated reovirus cores without adjuvant. Each mouse was sacrificed and their spleen cells were extracted. Fusion of the mouse spleen cells with SP2/0 myeloma cells was then performed and those cells that had fused were selected for and fusion was confirmed by ELISA (data not shown). Eleven of the ELISA positive antibodies were isotyped (Table 7) and were then cloned twice by the limiting dilution method and tested by ELISA (data not shown). Positive clones were allowed to grow to high numbers and spent media was produced by allowing the antibody-producing cells to die off. This spent media was used for all further experiments. An ELISA, with dissociated cores as the captured antigen, was performed to determine the approximate dilution of antibody required of each monoclonal (Table 7).

3.3.3 Characterization of α-Reovirus Core Monoclonal Antibodies by Western Blot

In order to begin the characterization of the specificity of each monoclonal antibody, a Western Blot was performed. Western Blot analysis demonstrates that only monoclonal antibodies #2-5C2 and #2-3C11 were Blot positive (Figure 17). Each of these two antibodies reacted with one of the λ proteins; however, we were unable to identify which λ by this method. A band of molecular weight corresponding to a λ dimer was also detected by Western Blot. The three λ proteins have very similar molecular

Table 7- Characterization of Reovirus Core Specific Monoclonal Antibodies

Antibody	Type	ELISA	Optimal Working Dilution for ELISA	Western Blot
# 1-3C11	κ, IgG2a	+	1:5000	-
14B10	κ, IgG2a	+	1:100	-
12G4	κ, IgG2a	+	1:10	-
11F2	κ, IgG2a	+	1:100	-
2B6	κ, IgG2a	+	1:500	-
# 2-5C2	κ, IgG2b	+	1:1000	λ2
# 2-3C11	κ, IgG2a	+	1:1000	λ2
4G2	κ, IgG2a, IgG3	+	1:1000	-
3F6	κ, IgG2a, IgG3	+	1:100	-
2D9	κ, IgG2a	+	1:500	-
1D10	κ, IgG2a	+	1:100	-

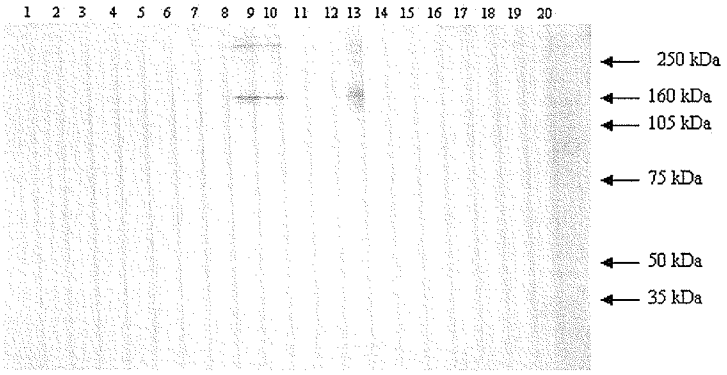

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
250 kDa
160 kDa
105 kDa
75 kDa
50 kDa
35 kDa

Figure 17. Western Blot Analysis of Reovirus Core Specific Monoclonal Antibodies. Dissociated reovirus cores were used to characterize the specificity of reovirus core specific monoclonal antibodies. A slot-blot apparatus allowed the characterization of all eleven antibodies at once. Lane 2: 12G4, Lane 3: 14D10, Lane 4: 2D9, Lane 5: 1D10, Lane 6: 3F6, Lane 7: #1-3C11, Lane 8: 4G2, Lane 9: #2-3C11, Lane 10: #2-5C2, Lane 11: 2B6, Lane 12: 11F2, Lane 13: Reovirus infected mouse serum as a positive control, Lane 14: Normal Mouse serum as a negative control, Lane 20: rainbow molecular weight marker. As can be seen in Lanes 9 and 10, #2-3C11 and #2-5C2 are specific for one of the reovirus λ proteins.

weights and do not separate well by SDS-PAGE. As well, our laboratory is lacking a polyclonal antibody which will identify all five core proteins in a Western Blot. A different method was required to identify the specificity of the monoclonal antibodies.

3.3.4 Characterization of α-Reovirus Core Monoclonal Antibodies by TGU Gel, Protein Elution and Western Blot

In order to characterize precisely the specificity of the Western Blot positive antibodies, #2-3C11 and #2-5C2, a method was required to obtain purified reovirus λ proteins. A Tris-Glycine-Urea gel was used to separate the three λ proteins (Figure 18) and the proteins were eluted from the gel by electro-elution. Purification of the eluted proteins was confirmed by mass spectrometry. A Western Blot was then performed with the λ proteins loaded in individual wells in order to characterize the specificity of the two Blot positive antibodies, #2-5C2 and #2-3C11. These blots indicated that both antibodies were specific for the λ2 protein (approximately 145 kDa) (Figures 19A and B) with both #2-3C11 and #2-5C2 demonstrating a minor amount of cross-reactivity with the λ1 protein which is visible on the original Western Blot membrane. Two bands are present on the Western Blot in lane 2; the higher molecular weight band corresponds to a λ2 dimer (approximately 290 kDa), the lower molecular weight band is the λ2 protein. This dimer does not appear in the dissociated cores used as a control, suggesting that the urea treatment dissociated the core particle and the protein complexes involved in its composition.

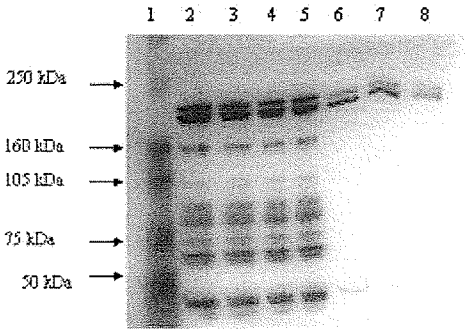
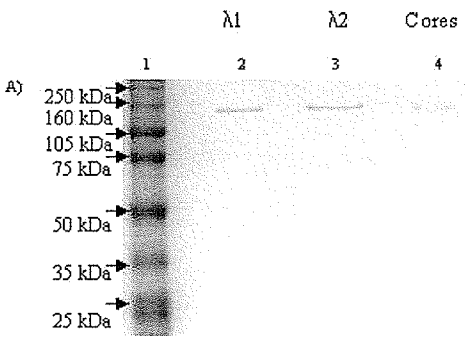
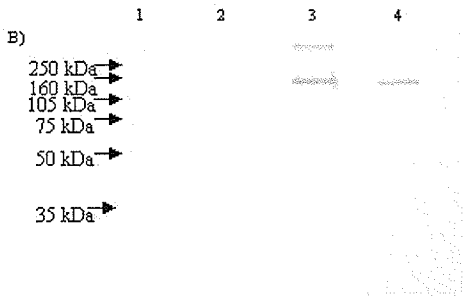
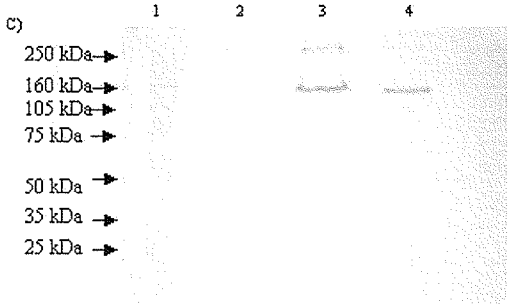

1 2 3 4 5 6 7 8
250 kDa
160 kDa
105 kDa
75 kDa
50 kDa

Figure 18. Tris-Glycine-Urea Gel Separation of Reovirus Core Proteins In order to separate the λ proteins, a TGU gel was used. Core proteins were loaded at various concentrations in order to optimize the amount of protein needed to obtain the ideal amount of separation. This gel is representative of the separation of the core proteins obtained by TGU-Gel electrophoresis. Lane 1: rainbow molecular weight marker, Lanes 2-5: non-dissociated, contaminated cores, Lanes: 6-8: dissociated, purified cores, 30 μg. The non-dissociated cores loaded in lanes 2-5 were purified in the same manner as the dissociated cores in lanes 6-8; however, the culture used to obtain the non-dissociated cores was contaminated with low levels of bacteria. The extra bands visible in lanes 2-5 are contaminating proteins.


λ1
λ2
Cores
1
2
3
4
A)
250 kDa
160 kDa
105 kDa
75 kDa
50 kDa
35 kDa
25 kDa


1
2
3
4
B)
250 kDa
160 kDa
105 kDa
75 kDa
50 kDa
35 kDa


1
2
3
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C)
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160 kDa
105 kDa
75 kDa
50 kDa
35 kDa
25 kDa

Figure 19. SDS-PAGE and Western Blot Analysis of Reovirus Core Specific Monoclonal Antibodies #2-3C11 and #2-5C2. In order to characterize the specificity of the blot positive antibodies, the λ proteins were purified by TGU gel, eluted by electro-elution and used in Western Blots. **A)** SDS-PAGE stained with Coomassie Blue demonstrating presence of proteins Lane 1: rainbow molecular weight marker, Lane 2: λ1, Lane 3: λ2, Lane 4: dissociated cores as a control. In lane 4, the λ proteins and σ2 were visible in the original gel. **B)** Western Blot characterizing #2-3C11 as specific for λ2. Lane 1: rainbow molecular weight marker, Lane 2: λ1, Lane 3: λ2, Lane 4: dissociated cores as a control. **C)** Western Blot characterizing #2-5C2 as specific for λ2. Lane 1: rainbow molecular weight marker, Lane 2: λ1, Lane 3: λ2, Lane 4: dissociated cores as a control. Both #2-5C2 and #2-3C11 demonstrated a minor amount of cross-reactivity with the λ1 protein which was visible on the original blot.

3.3.5 Characterization of α-Reovirus Core Monoclonal Antibodies by Immunoprecipitation Linked to Mass Spectrometry

A modified immunoprecipitation (IP) experiment linked to mass spectrometry was next employed in the attempts to characterize the monoclonal antibodies (Weiler *et al.*, 2003). A goat α- mouse antibody was coupled to sepharose beads and used to capture the α-reovirus monoclonal antibodies. The antibody-bead mixture was then incubated with dissociated cores, the beads were washed to eliminate any proteins that were not bound to the α-reovirus antibody and the protein was eluted from the antibody. Trypsin was used to digest the protein and mass spectrometry was used to identify the protein that had been bound to the antibody.

As this technique is novel and still in its development stage, a number of different factors needed to be optimized. Experiments determined that the concentration of both antibody and antigen did not appear to affect the results (data not shown). Cross-linking of the primary goat α-mouse antibody to the mouse α-reovirus antibody appeared to hinder antigen-antibody interactions, so cross-linking the antibodies was eliminated from the procedure. The stringency of the washes was also tested; a series of washes beginning with a harsh RIPA buffer wash, followed by a high salt, detergent-containing wash, a weak detergent wash and finally a PBS wash offers the cleanest mass spectrum (data not shown). The optimal incubation time of the primary antibody with the secondary antibody was determined to be 1 hour at room temperature on a rotator. Studies also demonstrated that the best results were obtained with liquid chromatography linked to tandem mass spectrometry, rather than solely analysis by MALDI-QqTOF. The fractions obtained in the LC-MS/MS were separated by hydrophobicity on a C_{18} column.

By this IP technique, proteins present after the incubation of antigen and antibody and the washing, were identified for two of the monoclonal antibodies, #1-3C11 and 4G2 using the computer program, Knexus. In the case of #1-3C11, the proteins identified were λ1, λ2 and σ2 (expectation values of $2.3x10^{-41}$, $3.0x10^{-39}$ and $8.2x10^{-9}$, respectively). These results suggest that these three core proteins may be forming a complex; however, this does not allow us to determine whether the antibody recognizes the complex of proteins or whether it recognizes an individual protein within the complex. Electron microscopy of whole cores and the dissociated, dialyzed cores suggests that the cores are dissociated but can not demonstrate the presence of complexes in the mixture (data not shown). Attempts to break up the complex by sonication and diluting out the high concentration of urea used to dissociate the cores just before incubation with the antibody were unsuccessful, giving results which suggest the complex is still intact. Another attempt to keep complexes apart was undertaken by leaving a low level of urea present during the incubation, this disrupted the antibodies as well and resulted in no positive identifications (data not shown).

3.3.5 Characterization of α-Reovirus Monoclonal Antibody Specificity by Immunoprecipitation Linked to SDS-PAGE and Mass Spectrometry

In another attempt to characterize the specificity of antibody #1-3C11, an immunoprecipitation was performed whereby the antibody was pulled down by Immunopure Protein A/G beads (Pierce), the antibody was then incubated with dissociated cores, washed with two different wash conditions (see section 2.6.6) and the bead/antibody/antigen complex was loaded on a gel (Figure 20). In-gel digestion and

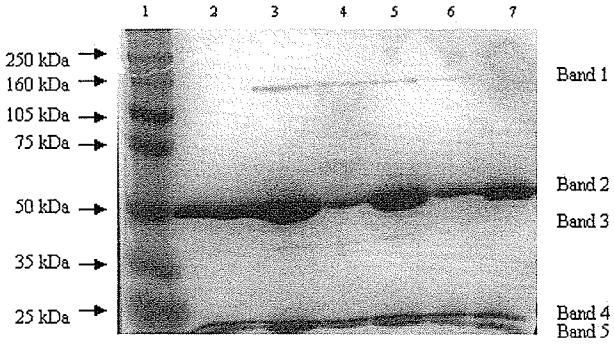
1
2
3
4
5
6
7
250 kDa
160 kDa
105 kDa
75 kDa
50 kDa
35 kDa
25 kDa
Band 1
Band 2
Band 3
Band 4
Band 5

Figure 20. Characterization of Reovirus Core Specific Monoclonal Antibody #1-3C11 by Immunoprecipitation. In order to characterize the specificity of one of the blot negative antibodies, the antibody was used to pull down protein from dissociated cores. The antibody was either directly coupled to sepharose beads or was captured by protein A/G beads coupled to sepharose beads. After incubation with the antigen, the antibody/ antigen complex was subjected to one of two sets of wash conditions: (1) 3 cycles with 100 mM BOG, 1% Triton X-100 in 1X PBS and 1X PBS (2) 3 cycles with 0.05% DOC, 1% Triton X-100 in 1X PBS and 1X PBS. Lane 1: rainbow molecular weight marker, Lane 2: A/G beads, #1-3C11 + PBS, Lane 3: A/G beads, #1-3C11 + dissociated cores (wash 1), Lane 4: coupled #1-3C11 + dissociated cores (wash 1), Lane 5: A/G beads, #1-3C11 + dissociated cores (wash 2), Lane 6: coupled #1-3C11 + dissociated cores (wash 2), Lane 7: #1-3C11. Bands 1-5 were excised, subjected to in-gel digestion and identified by mass spectrometry.

MALDI QqTOF mass spectrometry were then used to identify bands 1-5. The reovirus core proteins pulled down by #1-3C11 using this method were λ1 with an expectation value of 3.5×10^{-14} (24 peptides of 44 matched, 30% sequence coverage) (Band 1) and σ2 with an expectation value of 1.4×10^{-11} (14 peptides of 35 matched, 40% sequence coverage) (Band 3) (Figures 21 and 22). Bands 2, 4 and 5 were identified as immunoglobulin. These results are consistent with those found using the alternative immunoprecipitation experiment described in section 2.6.5 with the exception of the absence of the λ2 protein. LC-MS/MS was performed on the band identified as λ1 to determine if λ2 was present in the sample and demonstrated that only λ1 was present (expectation value 4.5×10^{-20}).

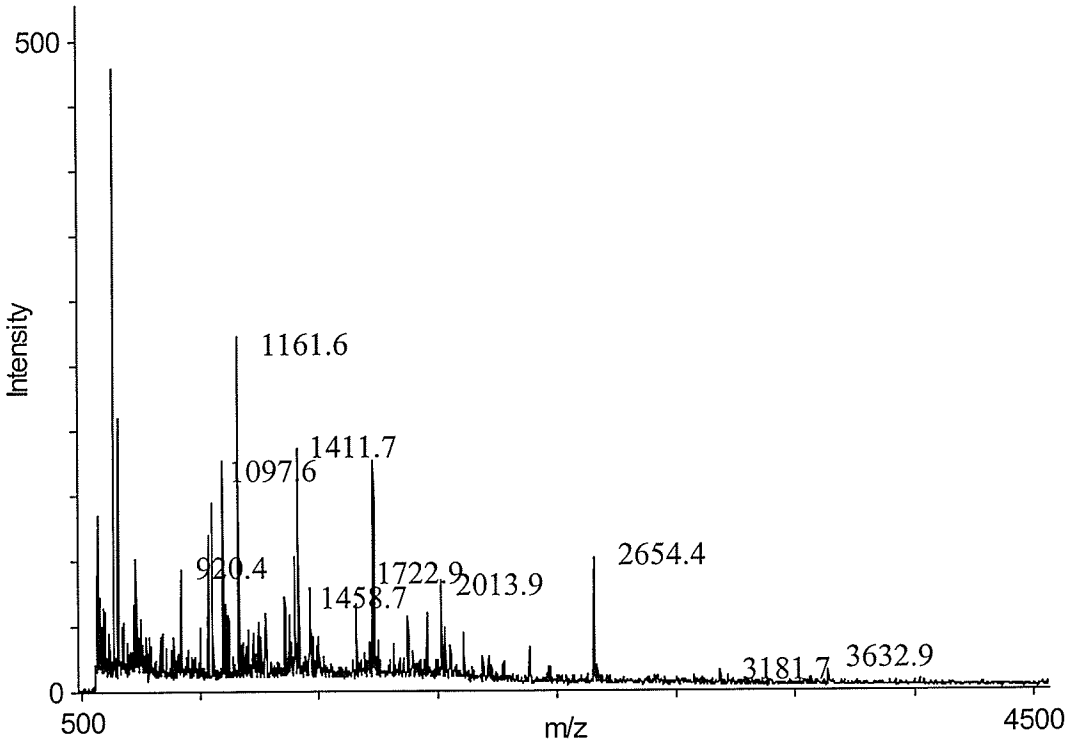
500
Intensity
0
500
m/z
4500
1161.6
1411.7
1097.6
920.4
1722.9
2013.9
1458.7
2654.4
3181.7
3632.9

Figure 21. Mass spectrum of λ1 pulled down by Reovirus Core Specific Monoclonal Antibody #1-3C11. Dissociated cores were incubated with #1-3C11, which had been directly coupled to sepharose beads or captured by protein A/G beads linked to sepharose beads. After incubation, the beads were washed and the beads/antibody/antigen complex was loaded on a 10% mini SDS-PAGE gel. The bands were excised and subjected to in-gel digestion. This sample corresponded to Band 1 (see Figure 20). Peptides produced during in-gel digestion were measured by MALDI QqTOF and analyzed against the NCBInr database using ProFound (Proteometrics). The protein in Band 1 was identified as λ1 with an expectation value of $3.5x\ 10^{-14}$, with 30% sequence coverage (24 of 44 peptides matched). Only a few representative peptides are labeled and shown here.

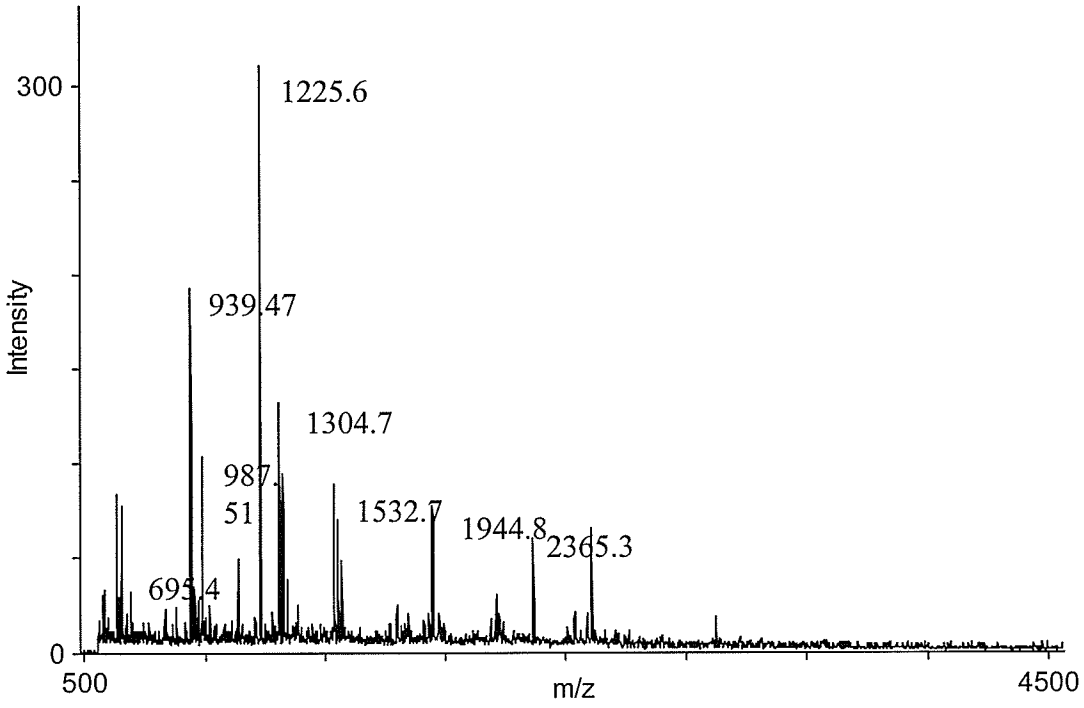
300
0
Intensity
1225.6
939.47
1304.7
987.
51
1532.7
1944.8
2365.3
695.4
500
m/z
4500

Figure 22. Mass spectrum of σ2 pulled down by Reovirus Core Specific Monoclonal Antibody #1-3C11. Dissociated cores were incubated with #1-3C11, which had been directly coupled to sepharose beads or captured by protein A/G beads linked to sepharose beads. After incubation, the beads were washed and the beads/antibody/antigen complex was loaded on a 10% mini SDS-PAGE gel. The bands were excised and subjected to in-gel digestion. This sample corresponded to Band 3 (see Figure 20). Peptides produced during in-gel digestion were measured by MALDI QqTOF and analyzed against the NCBInr database using ProFound (Proteometrics). The protein in Band 3 was identified as σ2 with an expectation value of 1.4×10^{-11}, with 40% sequence coverage (14 of 35 peptides matched). Only a few representative peptides are labeled and shown here.

4. Discussion

4.1 Sequencing of the ARV M Genes and μ Proteins

In an attempt to learn more about the MRV μ proteins, as well as the ARV proteins, sequencing of the three ARV μ proteins was undertaken. Previous research has provided the sequence of the ARV σ proteins (Duncan, 1999; Shmulevitz, 2002) and recently the sequence of the ARV strain S1733 λ1 protein was also published (Touris-Otero *et at.,* 2004). The sequencing of the M genes and μ proteins of ARV 138 and ARV 176 is part of an overall strategy to obtain the complete genomic set of these avian reovirus strains, as well as their predicted proteins. The sequence of the ARV M genes and μ proteins also provides valuable information regarding the structure and function of the μ proteins, which until now was predominantly speculative. By comparing the ARV M genes and their predicted encoded μ proteins with those of MRV which already have fairly well characterized activities associated with them, hypotheses can be drawn about the roles of the ARV proteins. As well, these comparisons can lead to highly conserved individual amino acids or regions of amino acids which may play important roles in the structure of the protein, its function or both. Alignments, BLAST searches and phylogeny can all contribute to identifying important regions of both the ARV and MRV μ proteins. In this way, the sequencing of the AVR μ proteins can be directly used to further our understanding of the ARV system, but it can be indirectly used to learn more about MRV, as well as some of the other aquareoviruses with homologous proteins identified during this study.

Sequencing of the ARV 176 and 138 M1 genes demonstrated approximately 90% homology which is comparable to the homology of the prototypic MRV Type 1 Lang and

Type 3 Dearing M1 genes. The ARV M1 gene was also found to be shorter than its MRV counterpart and to encode a protein slightly smaller than that in MRV. Interestingly the encoded proteins, μA in ARV and μ2 in MRV have very different isoelectric points (8.4 in ARV vs. approx. 7 in MRV) suggesting a high level of variation between the proteins. Alignment of the prototype MRV μ2 and ARV μA proteins demonstrate approximately 30% homology, predominantly in a conserved region which has previously been identified in MRV as containing a motif responsible for NTPase activity (Yin *et al*, 2004). The μ2 protein in MRV is a putative RdRp cofactor. The same motif is found in the ARV μA protein. BLAST searches of the NCBI database identified several other aquareoviruses (see Figure 6) which contain the same NTPase motif, suggesting that all 9 proteins may have the same activity. Each of these proteins also has the predicted "alpha-beta" secondary structure, suggesting that this secondary structure plays a role in maintaining the function of the protein. There are a number of conserved amino acids throughout the proteins suggesting that they may have similar functions within their respective viruses and that these amino acids play a crucial role in maintaining the function and structure of the protein.

Sequencing of the ARV M2 genes demonstrated approximately 90% homology within the ARV, similar to that seen with the M1 gene. The ARV M2 genes are predicted to encode a μB protein which is slightly smaller than the predicted equivalent MRV protein, μ1. Alignments of the MRV μ1 protein with the ARV μB protein demonstrate approximately 45% identity with several conserved regions throughout the protein. This level of conservation is higher than the 20-30% conservation seen for the other ARV μ and σ proteins which have been sequenced. As an outer capsid protein, one would expect

the divergence to be greater due to antibody selection; however, it is possible that the μB protein may be protected by its interaction with σ3 and the λ2 core protein. As well, multiple cleavages of the μB protein and conformational changes are required during viral entry into the host cell, as well as during assembly. These constraints may also play a role in limiting the evolution of the μB protein. BLAST searches identified three aquareoviruses with proteins containing similar motifs and primary amino acid sequence. Each of these proteins has the predicted "alpha-beta" secondary structure, suggesting that the secondary structure is crucial to the function of the protein. The MRV μ1 protein is the major outer capsid protein and plays a role in the penetration of the cell. The alignments of the ARV, MRV and aquareoviruses suggest that these proteins may have a similar role within the virus particle. The higher level of sequence similarity may be directly related to the role the protein plays in cellular penetration. The protein may interact with a host receptor during virus binding and penetration and the conserved regions in the μ outer capsid protein may reflect homologous regions of cell receptors required for binding by the orthoreovirus genus.

Sequencing of the ARV M3 genes demonstrated approximately 94% homology within the ARV, slightly higher than that seen with the other ARV M genes. The ARV M3 genes are predicted to encode a μNS protein which is significantly smaller (84 amino acids) than the predicted equivalent MRV μNS protein. Alignments of the MRV μNS protein with the ARV μNS protein demonstrate approximately 25% identity with predominantly conserved amino acids throughout the protein. BLAST searches did not identify any different species of reoviruses with similar proteins in GenBank; however, it did identify the ARV 1733 strain as having a similar protein. Alignment of the ARV 176

μNS with the ARV 1733 and ARV S1133 strains' μNS proteins suggests that these strains may be different isolates of the same virus since there have been no documented biological differences. Further genetic studies will be required to identify the relationship between these viruses. As well, the lack of homologous aquareovirus proteins may not be a lack of non-structural proteins in the aquareoviruses. This may be an artifact, due to the lack of complete aquareovirus genome sequences in GenBank. Perhaps the homologous protein remains to be sequenced in the aquareovirus family. Interestingly, when computer analyses were performed to determine the presence of currently recognized motifs in the ARV μNS, none were found. This was similar to what was seen with the ARV μA and μB proteins. However, when the Swiss-Model program (Swiss Institute of Biotechnology, Switzerland; Biozentrum, University of Basel, Switzerland) was used to predict the tertiary structure of the ARV μNS protein, Swiss-Model was able to predict a 3-D model suggesting that similar sequence or motifs had been entered into the program. The same program was unable to predict a tertiary structure for the ARV μA and μB proteins.

The goal of this study was to sequence the ARV M genes, analyze the predicted encoded μ proteins and compare these sequences with the already completed MRV gene and μ protein sequences to determine if there were any highly conserved regions which may play an important structural and functional roles. Conserved regions were identified in all three proteins that may be significant to the protein and the virus, as a whole. BLAST searches also provided similar aquareovirus proteins which added weight to the hypothesis of the conserved amino acids being crucial for the protein to function properly. These regions should be used as a starting point for mutational analyses to

determine the part each conserved region plays. Another reason this project was undertaken was that the only ARV genes which had been sequenced at the commencement of the project were the ARV S genes. Recently, the first ARV L gene sequence was published. It was our goal to sequence the M genes in an attempt to aid in completing the genome sequence for ARV. Once the genome has been completely sequenced, real understanding of how the virus functions and how it is unique from MRV and other reoviruses can begin. A significant addition to this study was the addition of proteomics to confirm the sequence obtained by traditional sequencing methods. There have been problems with reovirus sequences in the past, whereby sequences were published and later retracted due to insertions or deletions missed in the initial sequencing (Harrison *et al.*, 1999). Mass spectrometry can be used to confirm whether insertions or deletions are present in the sequence before it is released to the public. However, the technique can only be used for structural proteins which can be easily purified away from the other viral proteins. This is a minor problem and as this study demonstrates, the combination of proteomic approaches with traditional sequencing can provide solid sequence data.

4.2 Characterization of Reovirus Core Specific Monoclonal Antibodies

Antibodies are essential tools in molecular biology which can be used in a wide variety of ways, including Western Blots, ELISAs and immunoblotting. As mentioned previously, antibodies are a class of proteins which have specific binding properties and can be raised against both natural and synthetic compounds. In the laboratory, antibodies tend to be used as a marker to detect the presence of protein, as well as being used for protein purification by immunoaffinity. In order to execute these methods, large amounts

of a specific antibody are required. This is of particular importance in our laboratory where we study the structure and function of reovirus proteins, yet lack monoclonal antibodies to the majority of the virus proteins. This lack of antibodies is a hindrance in the attempts to identify and generate purified protein in our laboratory. The generation of a hybridoma allows for the production of a continuous supply of an antigen-specific monoclonal antibody. However, in the past, the generation and characterization of a hybridoma has required purified protein or recombinant DNA. The novel technique combined with traditional immunological methods explored in this study eliminates the need for purified protein and created reovirus core specific monoclonal antibodies from dissociated core proteins. The use of a mixture of proteins allowed for the generation of a large number of hybridomas whose specificity was then characterized using mass spectrometry combined with Western Blots, immunoprecipitation and ELISA. After this characterization, the antibodies can now be used in our laboratory as molecular tools to continue our studies of the reovirus proteins.

This study originally had two basic goals. The first was to characterize a number of reovirus core specific monoclonal antibodies and the second was to optimize an immunoprecipitation experiment (IP) linked to mass spectrometry (Weiler *et al.* 2003) devised by our collaborators in Dr. John Wilkins' lab (University of Manitoba, Dept. of Immunology). The basic procedure for the experiment involved the capturing of the mouse α-reovirus monoclonal antibody by goat α-mouse antibody bound to sepharose beads. Dissociated cores were then incubated with the antibody for an hour, the supernatant was removed and the beads were washed to remove all unbound antigen. The antigen bound to reovirus antibody was then digested with trypsin and mass spectrometry

was used to identify the antigen. However, the process required a number of manipulations and optimization before any positive identifications were made. By varying the number of core particles, I was able to demonstrate that 10^{13} core particles are required to pull out the protein of interest by this method. This assumed that there were approximately 10^{15} copies of the highest copy number proteins. This was comparable to the protein concentrations found to be optimal by Weiler *et al* (2003). The amount of antibody required for efficient antigen capture was also optimized by IP and it was determined that 1 mL of spent media is sufficient, concentrating the antibody does not enhance antigen capture. I also attempted to vary the conditions under which the antibody and antigen were incubated and found that 1 hour at room temperature is optimal. When I started this IP protocol, I would incubate the anti-reovirus antibody with the beads overnight on a rocker at 4°C. To determine whether that was producing the best antibody capture, I also tried incubating the beads and antibodies for 1 hour on a rocker at room temperature. When comparing the results obtained from each experiment, positive IDs were obtained more consistently when the antibody was incubated with the beads at room temperature for an hour. Antibodies are composed of proteins and at 4°C the reaction kinetics are slowed like other proteins. It is possible that incubation overnight at 4 °C does not allow the amount of α-reovirus antibody capture that can be obtained by incubation at room temperature for 1 hour. As well, the incubation at 4°C may hinder the function of the α-reovirus antibodies so they are unable to capture antigen as well as those α-reovirus antibodies incubated at room temperature.

A number of problems still remain to be resolved with this experiment. Initially, I cross-linked the goat anti-mouse antibody coupled to the sepharose beads to the reovirus

antibody using 10 mM DMP for 1 hour at room temperature. In the literature people use a wide variety of concentrations of DMP and DSS for cross-linking and the cross-linking time varies from 10 minutes to 1 hour. I attempted cross-linking with 20 nM, 200 nM and 2000 nM DMP and DSS for 10 minutes to 1 hour and never obtained a successful ID after cross-linking. Without crosslinking, I obtained positive IDs each time after the antibody had been extracted. This was likely because the concentration of crosslinking agents used was too high or the incubation period with the agent was too long. It is possible that either the activity of the antibody was being damaged by the crosslinking agent or that the agent was too effective and was crosslinking all available binding sites on the exposed antibodies, in which case there were no sites available for the binding of antigen. Another issue that remains to be resolved is the wash conditions required after incubation of antibody and antigen. The exact combination of detergents required to break up any complexes that may have formed between the proteins of the dissociated cores and to wash away any unbound proteins has not yet been completely resolved.

The IP method described above was used to characterize the antibody #1-3C11. LC-MS/MS was used to identify the antigen pulled down by this antibody and these proteins were identified as $\lambda 1$, $\lambda 2$, and $\sigma 2$. The wash conditions were varied in an attempt to break up what appeared to be complex but in each attempt of this IP protocol these three proteins were pulled down. The expectation values for each protein clearly demonstrated that each protein was present in the mixture (2.3×10^{-41}, 3.0×10^{-39} and 8.2×10^{-9}, respectively). The score reflects the numbers of peptides matched compared with the number of peptides measured and an accurate quantitation of each protein present can not be determined by this method. This data suggests that these proteins may

be forming a complex; what remains unknown is whether the antibody recognizes the complex or an individual protein within the complex. A question that remains is when this complex of λ1, λ2, and σ2 is being formed. Electron microscopy suggests that the cores were dissociated in 9M urea however a native PAGE gel could corroborate that information. Electrospray mass spectrometry allows the study of protein folding, conformational changes and protein-protein interactions. This technique could be performed on core particles before dissociation, after dissociation, before incubation with the antibody and after incubation with the antibody to determine if and when the proteins form complexes. In this way we could track the dissociation of the cores and any complexes present.

The primary objective of this study was to characterize the reovirus core specific monoclonal antibodies by IP linked to mass spectrometry. The antibody #1-3C11 is Blot negative, suggesting that it recognizes a conformational epitope. A different type of immunoprecipitation experiment was attempted to clarify the results already obtained by IP. The antibody was coupled to beads or captured by protein A/G bound to sepharose bead and incubated with dissociated cores. After washing the beads, the beads, antibody and antigen were loaded onto a gel and in-gel digestion was performed on the bands. In this way, λ1 and σ2 were identified as present. LC- MS/MS was performed on the band identified as λ1 to determine if λ2 was present but only λ1 was present. This suggests that λ1 and σ2 may form a complex but does still not reveal the precise specificity of the antibody. It is possible that the wash conditions remain insufficient to remove the non-covalently associated proteins after the binding of λ1 to the antibody. In order to identify whether the antibody recognizes one protein or the whole complex, each of the three

proteins identified will need to be purified in their natural conformation and an ELISA can be performed to determine the precise specificity of #1-3C11. For now, the specificity of this antibody needs further clarification.

Currently, our laboratory does not possess purified or recombinant MRV core proteins. The IP experiment linked to mass spectrometry was undertaken in an attempt to characterize antibodies made from a mixture of all five core proteins without requiring the individual proteins. At the present time, the IP protocol is inconsistent in providing positive protein identifications and does not provide any identifications at all for the majority of the antibodies. Conditions for the protocol continue to require optimization such that the protocol works with any antibody of interest and consistent protein identifications are given. So while conventional approaches to identify the specificity of an antibody which require purified or recombinant proteins, such as ELISA, Western Blots, immunoprecipitations, may currently be the best method, I believe that with some work the IP protocol will have a greater sensitivity and be a much faster and more efficient way of characterizing antibodies.

In order to characterize the two Blot positive antibodies, the λ proteins were eluted from a TGU gel, their identity and purity was confirmed by mass spectrometry and the proteins were used in a Western Blot with #2-5C2 and #2-3C11 as probes. The specificity of both antibodies was identified as λ2. The specific epitopes that each antibody recognizes are likely linear as the epitopes are recognized in a Western Blot when the proteins have been denatured by heat, SDS and β-mercaptoethanol. Antibody #2-3C11 and #-25C2 both appear to have minor amounts of cross-reactivity with the λ1 protein (visible on the original blot); however, it primarily recognizes the λ2 protein.

Now that the specificity of these antibodies has been characterized, they can be used in our laboratory to purify and identify the λ2 protein in a wide variety of different molecular protocols.

4.3 Conclusions

In this study, the ARV 176 and ARV 138 M1, M2 and M3 genes were sequenced by traditional sequencing methods. The predicted proteins encoded by the three genes, μA, μB and μNS, were then analyzed using a variety of computer programs to determine their primary amino acid sequence, and predict secondary structure, isoelectric points, and motif homology with other proteins. Mass spectrometry was used to confirm that there were no insertions or deletions present in the sequence data obtained for the ARV structural μ proteins, μA and μB. The gene and protein sequences of the ARV were compared, as were the ARV and MRV to determine conserved regions and individual amino acids which play important structural and functional roles within the protein. Alignment analysis suggests that the ARV and MRV μ proteins may have similar functions within their respective virus particles. BLAST searches were also performed to identify other proteins with similar motifs and sequences and for the μA and μB proteins, a number of aquareovirus proteins were identified which may have similar functions to that of the ARV and MRV proteins. The amino acids identified by this comparative analysis will provide useful places to start in identifying the regions of the μ proteins responsible for their function.

The second part of this study involved the characterization of reovirus core specific monoclonal antibodies. The antibodies were produced by the injection of dissociated cores, rather than with purified protein. Eleven monoclonal antibodies were

identified as core protein positive by ELISA and two of these were identified as Western Blot positive (#2-5C2 and #2-3C11). The two Blot positive antibodies were λ positive; in order to confirm the specific λ, a TGU gel was used to purify the λ1 and λ2 proteins. The proteins were eluted from the gel and a Western Blot was used to determine the specificity of the Blot Positive antibodies. In this manner, both #2-5C2 and #2-3C11 were identified as being specific for λ2, with minor amounts of cross-reactivity for λ1. Attempts were made to purify the λ3 protein; however, due to its low copy number, purification of the protein was unsuccessful. It is unlikely that #2-5C2 and #2-3C11 interact with the λ3 protein beyond a minor amount of cross-reactivity given that each antibody strongly reacts with the λ2 protein in a Western Blot.

An attempt was made to characterize one of the Western Blot negative antibodies (#1-3C11) using a modified immunoprecipitation experiment linked to mass spectrometry designed by Weiler *et al.* (2003). Many modifications were made to the protocol in an attempt to optimize the conditions; however, three core proteins, λ1, λ2 and σ2 were always pulled down with the antibody. An immunoprecipitation using in-gel digestion was also attempted using #1-3C11 and λ1 and σ2 were also pulled down. It remains to be determined whether the core proteins form a complex after the core protein has been dissociated, whether the core proteins are ever truly dissociated or whether the proteins are just strongly associated with one another and with the right wash conditions they can be separated. Once the question of the protein complex has been answered, the question as to whether #1-3C11 recognizes an individual protein or a complex can be answered.

4.4 Future Directions

As with most studies, these experiments raise more questions than they answer. With regard to the sequencing of the ARV M genes and analysis of the predicted μ proteins, there are many avenues which can now be pursued to further our understanding of this virus. The comparisons of the ARV and MRV μ proteins with several aquareovirus homologous proteins identified a number of highly conserved individual amino acids and regions of amino acids which may play important structural and functional roles within the three μ proteins. Mutational studies should be performed in both MRV and ARV to determine the roles of these amino acids and whether they are actually important to protein function. This mutational analysis could be performed by insertions or deletions into the genome or by point mutations. It would also be interesting to determine whether it is the exact amino acid that is important or whether the amino acid could be replaced with one of similar size or hydrophobicity, for example, with the protein maintaining its structure and function within the virus particle and if infectious virus continued to be produced.

Within the ARV system, there remains much to be learned about the virus. Functional studies should be performed with the μ proteins to determine if they have similar activity in ARV that they do in MRV, whether the protein may have other activity in ARV than in MRV and whether the proteins may play a similar role within their respective viruses. ARV tend to grow to lower titres than MRV and obtaining a large amount of virus is difficult; however, it would be quite advantageous if enough ARV could be purified for X-ray crystallography to compare with the crystal structure of the MRV particle. A cloning system in our laboratory which would allow us to clone and

purify the ARV μ proteins would aid in structural and functional studies. A useful tool which will be required for obtaining purified protein needed for these types of studies will be monoclonal antibodies specific for ARV proteins. Currently, our laboratory has an ARV polyclonal antibody but is lacking monoclonals. Perhaps when all the MRV specific core monoclonal antibodies are characterized, the next step can be the production and characterization of ARV antibodies.

A lot of work remains to be done with the MRV core specific monoclonal antibodies. With the two Blot positive λ2-specific antibodies characterized in this study, it would be worthwhile to perform ELISAs and Western Blots using whole virus and cores since the λ2 protein is exposed on the outside of the virus particle and may be able to interact with the antibody in the virus' native form. Immunofluorescence studies using #2-3C11 and#2-5C2 to probe virus, ISVP and cores would also be interesting. Epitope mapping could be performed to determine the specific epitope recognized by each antibody; however, this will require purified core proteins. Neutralization studies and competition assays could also be performed to further characterize the affinity and specificity of the antibodies. A previously characterized λ2- specific monoclonal antibody, 7F4, (Virgin *et al.*, 1991) could be included in comparative studies of #2-5C2 and #2-3C11, e.g. competition and affinity assays, in order to determine the most effective α-λ2 antibody. While this study began with eleven monoclonals and then further narrowed it down to two, there are almost 100 hybridomas that still need to be cloned and characterized. Of the eleven antibodies that I began with, only 2 have been characterized. The remaining 9 are all Western Blot negative, suggesting that they may recognize conformational epitopes. In order to characterize these antibodies, purified

proteins in their native confirmation will be required. A cloning system is currently being set up in our laboratory to produce purified proteins which can be used to coat ELISA wells and quickly identify the specificity of the antibody. It may also be possible to renature the proteins eluted from the TGU gel by slowly dialyzing them against decreasing concentrations of urea. The renatured proteins could also be used in an ELISA or may also be used in a native gel and a Western Blot to identify the specificity of the antibodies which remain uncharacterized. However, a large quantity of dissociated cores would be required for the renaturation of proteins and the renaturation conditions would need to be optimized for each core protein. Once a cloning system has been developed for each of the MRV reovirus proteins, large quantities of purified protein will be readily accessible without the need for purified virus and the denaturation and renaturation process required by the TGU protein purification method.

The IP experiment linked with mass spectrometry continues to need optimization and experimenting with different conditions. The question of whether the dissociated core proteins reform a complex following dialysis of the urea needs to be answered before any more conditions are altered in the IP protocol. As mentioned above, samples of the dissociated cores could be taken before dissociation, after dialysis, before and after incubation with the antibody and electrospray mass spectrometry could be used to determine whether complexes are present. Once this question has been answered, the wash conditions for the IP can be further refined and the IP could likely then be used to identify the specificity of the other antibodies. The modified IP using in-gel digestion to identify the proteins pulled down by the reovirus antibodies was a fast and efficient way of performing the same type of experiment and may also be a useful method to

characterize the reovirus core specific monoclonals when the complex formation question is answered. Once all of these antibodies have been characterized, our laboratory will hopefully have a full complement of core protein specific monoclonal antibodies at our disposal that can be used in studies for years to come.

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